Collagen Production by Chondrocytes in Alginate Bead Culture

Kate Elizabeth Gregory

Doctor of Philosophy

University of Edinburgh

1997

CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABSTRACT

Articular cartilage is a connective tissue that covers the ends of long bones and is noted for its resilience and mechanical durability. These properties are mainly due to a highly ordered extracellular matrix (ECM) produced by chondrocytes and is composed of a network of collagen fibrils in which other components (e.g. proteoglycans) are embedded. As *in vivo* studies on chondrocyte differentiation and ECM interactions have proven difficult, *in vitro* cell culture systems have been developed to study these processes. For these, support matrices are required to maintain chondrocyte phenotype and the traditional approach has been to use agarose gels, although isolation of matrix components from these gels is relatively difficult. Alginate beads have therefore been introduced as a recent alternative to agarose because they dissociate in the presence of a calcium chelator, allowing rapid recovery of chondrocytes and ECM proteins.

This thesis addresses the question "*Do alginate beads provide an environment similar to that of cartilage for the culture of chick chondrocytes*?" and answers were obtained using a range of biochemical and other techniques to compare the ECM constituents produced in alginate to those observed *in vivo*.

The results show that, by most criteria, the alginate system provides an environment similar to that *in vivo*: cartilage-specific collagens (II, IX, X and XI) were produced, with ratios analogous to those *in vivo,* and collagen IX was present in both proteoglycan and non-proteoglycan forms. Moreover, sulphated glycosaminoglycans were retained in the cell-associated matrix over a 14 day culture period, an observation that suggests proteoglycan aggregation.

Two major differences were however noted between alginate culture and cartilage. First, collagen failed to assemble into fibrils, but instead formed segment-long-spacing (SLS) crystallites, a form normally found when collagen assembles under low pH conditions, and here the failure in assembly was shown to be due to the presence of alginate. Second, a substantial proportion of procollagen was not fully processed to collagen, leaving high amounts of a precursor which retains the N-

propeptide (pN II). Substitution of 10% fetal bovine serum (FBS), which contains proteinase inhibitors, with insulin in the culture medium decreased pN II concentrations (relative to collagen II) but also decreased total collagen production. The enzyme procollagen N-proteinase (107 kDa), which cleaves N-propeptides from pN-collagen I, was active on pN II when a mixture of ECM components was present but was not active on a purified pN II substrate. This suggested that the enzyme requires a co-factor for activity on pN II, but preliminary investigations failed to identify it.

This thesis thus shows that alginate beads provide a good environment for studying chondrocyte differentiation and ECM production, but not for morphological studies.

DECLARATION

I declare that all material presented in this thesis, unless stated otherwise, is the sole work of the author, as is the composition.

Kate E. Gregory

ACKNOWLEDGEMENTS

I would like to thank my supervisors Dr David Hulmes and Dr Jonathan Bard for all their help and advice over the past 3 years, particularly in the writing of this thesis. I would also like to thank the following people:

the original members of Lab 334, especially Mark Marsden and Ian Purdom for their expertise and Janet Anderson-McKenzie for the collagen II and IX antibodies; the people in Lyon, especially Dr. Jean Farjanel and Dr. Elizabeth Aubert-Foucher for the cartilage collagens and collagen XIV respectively; the Bruckner lab in Munster, especially Ulrich Blaschke and Dr. Erik Hedbom for the cartilage collagens and the collagen X antibody respectively; Dr. Alain Colige for the N-proteinase; Derek Notman and Oonagh Gray for their expertise in electron microscopy; and many thanks to Dr. Sutherland Maciver for giving me somewhere to work when I was homeless!!.

I would also like to send out huge appreciations to all the people who bought me beer, took me out dancing and listened to me whinge over the past three years, particularly Harry and Jan.

Finally, I would like to thank the members of my family, especially Mum, Dad and Fiona for their patience, support and the bottle of Bolly!!.

ABBREVIATIONS

βAPN	β-aminopropionitrile
BCA	Bicinchoninic acid
BMP-1	Bone morphogenetic protein-1
BSA	Bovine serum albumin
CHAPS	(3-[(3-cholamidopropyl)-dimethylammonio]-1-propane-sulfonate)
CM	Cell-associated matrix
CO_2	Carbon dioxide
COL	Collagenous domain
cpm	Counts per minute
CS	Chondroitin sulphate
DEAE	Diethylaminoethyl
dH_2O	Distilled water
DMB	1,9-dimethylmethylene blue
DMEM	Dulbeccos modified eagles medium
DNA	Deoxyribonucleic acid
DS	Dermatan sulphate
ECM	Extracellular matrix
FBS	Fetal bovine serum
FITC	Fluoroscein isothiocyanate
FRM	Further-removed matrix
g	Relative centrifugal force
GAG	Glycosaminoglycan
^3H	Tritium
H_2O_2	Hydrogen peroxide
HCl	Hydrochloric acid
HPLC	High performance liquid chromatography
hr	Hour
hrs	Hours
HRP	Horse radish peroxidase
HS	Heparan sulphate
KS	Keratan sulphate
$MgCl_2$	Magnesium chloride
min	Minute
mins	Minutes
mRNA	Messenger ribonucleic acid
μCi	Microcurie
NaOH	Sodium hydroxide
NC	Non-collagenous domain
NEM	N-ethylmaleimide
OPD	O-phenyldiamine dihydrochloride
PBS	Phosphate buffered saline
pC-collagen	Collagen that retains the C-propeptide
PEG	Polyethyleneglycol
PG	Proteoglycan

pI	Isoelectric point
PMSF	Phenylmethylsulphonyl fluoride
pN-collagen	Collagen that retains the N-propeptide
pN II	Collagen II that retains the N-propeptide
PS	Penicillin-streptomycin
PTA	Phosphotungstic acid
rER	Rough endoplasmic reticulum
RNA	Ribonucleic acid
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SGAG	Sulphated glycosaminoglycan
SLS	Segment-long-spacing aggregate
TBS	Tris buffered saline
TBST	Tris buffered saline-Tween
TCA	Trichloroacetic acid
TEM	Transmission electron microscopy
TEMED	N',N',N',N', tetramethylethylenediamine
TES	N-[tris(hydroxymethyl)methyl-2-amino]-ethanesulphonic acid
TGFβ	Transforming growth factor β
Tris	Tris(hydroxymethyl)aminomethane
Tween-20	Polyoxyethylenesorbitan monolaurate

AMINO ACIDS

Single letter code	Three letter code	Amino acid
A	Ala	Alanine
C	Cys	Cysteine
D	Asp	Aspartic acid
E	Glu	Glutamic acid
F	Phe	Phenylalanine
G	Gly	Glycine
H	His	Histidine
I	Ile	Isoleucine
K	Lys	Lysine
L	Leu	Leucine
M	Met	Methionine
N	Asn	Asparagine
P	Pro	Proline
Q	Gln	Glutamine
R	Arg	Arginine
S	Ser	Serine
T	Thr	Threonine
V	Val	Valine
W	Trp	Tryptophan
Y	Tyr	Tyrosine

CHAPTER 1

INTRODUCTION

1.1 EXTRACELLULAR MATRIX

The extracellular matrix (ECM) is an organised meshwork that surrounds cells in connective tissues and contains collagens that provide tensile strength, proteoglycans and elastin that allow matrix resiliency and glycoproteins that create tissue cohesiveness (Hay, 1991). Collagen molecules assemble to form complex patterns which, along with the other ECM components, determine the mechanical properties of a wide diversity of tissues and organs. The loosely packed, but highly organised arrangement of collagen fibrils in cartilage, for example, is likely to involve interactions of the fibrillar collagens with fibril-associated collagens and/or proteoglycans (Brodsky and Eikenberry, 1985). The cells that produce these macromolecules influence matrix assembly and continuously interact both with the ECM they produce, as well as with the ECM produced by other cells that they contact (Stockwell, 1979). Matrix receptors, known as integrins, present on the cell surface link the ECM to the cell interior and many cell properties are thus dependent on the composition and organisation of the ECM (Horwitz, 1997).

This thesis investigates the collagens produced by chondrocytes cultured in alginate beads and thus this review starts with a brief summary of our current knowledge of the growing collagen "superfamily" and their biosynthesis. The review goes on to outline the structure and function of cartilage with a more detailed examination of the cartilage collagens and their interactions with other ECM components. Special reference is given to diseases of cartilage, as most of these involve mutations of collagen genes that lead to a breakdown of the collagen network.

1.2 COLLAGEN

The term collagen derives from the Greek, κολλα, glue and γενος, birth and was originally coined to designate the constituent of connective tissue that produces glue (Kielty *et al.,* 1993). In the 19th century, connective tissue was shown to be comprised of fibres but it took until the 1920's for the tissue to be solubilised and shown by electron microscopy and x-ray diffraction that the fibres were collagen

fibrils. This collagen type, that contains 3 α-chains, is now referred to as type I and is the major constituent of skin and bone (Kadler, 1994). A different α1 chain was then identified in chick cartilage and, as it was not present in skin or bone, became known as collagen II (section 1.3.3.1; Miller and Matukas, 1969). It soon became clear that there were further collagens as collagen III was discovered in skin (Epstein, 1974), collagen IV was identified in basement membranes (Kefalides, 1971) and collagen V in human placenta and adult skin (Rhodes and Miller, 1978). To date, 19 types of collagen have been identified in a wide range of tissues (Table 1.1; for reviews see van der Rest and Garronne, 1991; Hulmes, 1992; Mayne and Brewton, 1993; Kadler, 1994; Prockop and Kivirikko, 1995).

Collagen is now defined as "a structural protein of the extracellular matrix (ECM) which contains one or more domains having the conformation of a collagen triple helix" (van der Rest and Garronne, 1991) and is the single most abundant protein in mammals, accounting for 30% of all proteins (Burgeson and Nimni, 1992).

1.2.1 Classification of Collagen Types

The collagen "superfamily" can be divided into several classes on the basis of their polymeric structures or related structural features, with all members containing triple helical domains (Hulmes, 1992). Collagen types are distinguished by Roman numerals while different chains are designated by Arabic numerals. The key families are:

- fibrillar collagens (types I, II, III, V and XI)

  Collagens of this family form fibrils that have a characteristic 64-67 nm (D) repeating banding pattern when viewed by electron microscopy. The banding pattern arises from the staggered arrangement of individual molecules in the fibril (Figure 1.2; Kadler, 1994). These collagens can associate to form heterotypic fibrils and co-assembly of different combinations of these collagens results in tissue-specific fibrils such as collagens I and V in the cornea, collagens I and III in

TYPE	*CHAINS*	*SUPRAMOLECULAR STRUCTURE*	*TISSUE DISTRIBUTION (EXAMPLES)*
I	α1(I), α2(I)	fibrillar	most connective tissues
II	α1(II)	fibrillar	cartilage, vitreous humour
III	α1(III)	fibrillar	skin, tendon, aorta, lung
IV	α1(IV), α2(IV), α3(IV), α4(IV), α5(IV), α6(IV)	non-fibrillar meshwork	basement membrane
V	α1(V), α2(V), α3(V).	fibrillar	bone, cornea, etc.
VI	α1(VI), α2(VI), α3(VI)	5 nm banded fibrils	most connective tissues
VII	α1(VII)	anchoring fibrils	basement membrane (skin, oral mucosa, cervix)
VIII	α1(VIII), α2(VIII)	hexagonal lattice structure	Descemets membrane, endothelial cells
IX	α1(IX), α2(IX), α3(IX)	FACIT	cartilage, vitreous humour
X	α1(X)	hexagonal lattice structure	hypertrophic and mineralising zones of cartilage
XI	α1(XI), α2(XI), α3(XI)	fibrillar	cartilage, corneal stroma
XII	α1(XII)	FACIT	collagen I-containing tissues, cartilage
XIII	α1(XIII)	transmembrane	most connective tissues
XIV	α1(XIV)	FACIT	collagen I-containing tissues, cartilage
XV	α1(XV) only?	multiplexin	most connective tissues
XVI	α1(XVI) only?	FACIT	skin, smooth muscle, amnion
XVII	α1(XVII) only?	transmembrane	basement membranes, cornea
XVIII	α1(XVIII) only?	multiplexin	widespread, most abundant in liver and lung
XIX	α1(XIX) only?	FACIT	rhabdomyosarcoma cell line

Table 1.1 Vertebrate collagens
References: collagens I-XII, Hulmes, 1992; collagen XIII, Pihlajaniemi and Tamminen, 1990; collagen XIV, Castagnola *et al.,* 1992; collagens XV and XVIII, Rehn and Pihlajaniemi, 1996; collagen XVI, Pan *et al.,* 1992; collagen XVII, Gordon *et al.,* 1997; collagen XIX, Inoguchi *et al.,* 1995.

skin, and collagens II and XI in cartilage (section 1.3.3.2; Birk *et al.,* 1988; Mendler *et al.,* 1989; Fleischmajer *et al.,* 1990).

- fibril-associated collagens (types IX, XII, XIV, XVI and XIX)

  Members of this group are termed Fibril Associated Collagens with Interrupted Triple helices (FACIT; Shaw and Olsen, 1991). As their name suggests, these molecules contain collagenous domains interspersed by non-collagenous regions and they interact with the surface of collagen fibrils. Collagen IX is found covalently cross-linked to collagen II fibrils (section 1.3.3.3; Figure 1.5), while collagens XII and XIV are found in close association with collagen I fibrils in dense connective tissues (Dublet and van der Rest, 1991; Keene *et al.,* 1991; van der Rest and Dublet, 1996). Collagen XVI is the only member of this group whose non-collagenous domains contain cysteine residues which may be crucial to its stability (Pan *et al.,* 1992). Collagen XIX is the newest member of the "superfamily" to be identified and has been shown to undergo alternative splicing (Inoguchi *et al.,* 1995). The supramolecular assemblies formed by collagens XVI and XIX remain unknown as they have thus far only been described at the cDNA level.

  It has been proposed that all FACIT collagens have a role in stabilising the structure of the matrix by forming a link between fibrils and other ECM components, so mediating deformability of the fibril bundles (van der Rest and Dublet, 1996).

- network-forming collagens (types IV and VII)

  These collagens are found close to, or are part of, basement membranes. Collagen IV has a major role in support of the basement membrane and the assembled meshwork that it forms endows the membrane with a size-selective filtration property (Glanville, 1987). Collagen VII is the largest collagen identified so far and is a major component of anchoring fibrils which link the basement membrane to the underlying stroma (Burgeson, 1987).

- beaded filament collagen (type VI)

  This collagen type is essentially a glycoprotein with a short collagenous central domain and has a widespread distribution e.g. skin, liver and placenta. It assembles into a unique supramolecular structure of 5nm diameter microfibrils with a periodicity of approximately 100nm (section 1.3.3.5; Timpl and Engel, 1987).

- short chain collagens (types VIII and X)

  These collagens are small with their collagenous domains being about half that of fibrillar collagens. Collagen VIII is the major collagen of Descemet's membrane, the specialised basement membrane found around corneal epithelial cells, where it assembles into an hexagonal lattice (van der Rest and Garronne, 1991). Collagen X has a restricted distribution, being synthesised around hypertrophic chondrocytes during the process of endochondral ossification. Like collagen VIII, it forms an hexagonal lattice but has also been found associated with collagen II fibrils (section 1.3.3.4; Chen *et al.*, 1990; Kwan *et al.*, 1991).

- transmembrane collagens (types XIII and XVII)

  A new group of collagens that has recently emerged is the transmembrane family (Rehn and Pihlajaniemi, 1996). These collagens are an inherent part of the cell membrane although they have large extracellular domains. They therefore have a potential role in signal transduction from both the outside-in and the inside-out. Both members have been shown to undergo alternative splicing. Collagen XIII consists of three collagenous and four non-collagenous domains and locates to the plasma membrane of cultured fibroblasts (Rehn and Pihlajaniemi, 1996). The extracellular domain of collagen XVII binds to basement membranes, whereas the intracellular domain contributes to the hemidesmosome (Li *et al.*, 1993; Hopkinson and Jones, 1996; Gordon *et al.*, 1997).

- multiplexins (types XV and XVIII)

  Another new subgroup of collagens has been termed the multiplexins (Multiple triple helix domains with interruptions; Rehn and Pihlajaniemi, 1996). The genes

encoding these collagens are highly homologous indicating a common ancestor but, as they have only recently been discovered, their functions are still to be elucidated (Huebner *et al.,* 1992). Collagen XVIII has been shown to be mainly expressed by liver hepatocytes, endothelial cells and certain epithelial cells (Saarela *et al.,* 1996).

1.2.2 Collagen Biosynthesis

The production of collagen molecules is a complex biosynthetic process with most of the knowledge being derived from studies on the fibrillar collagens (Figure 1.1; Burgeson and Nimni, 1992; Hulmes, 1992; van der Rest *et al.,* 1993; Kadler, 1994). Intracellular events include gene transcription, mRNA translation, post-translational modifications, monomer assembly, molecular packaging in vesicles and, following fusion with the cell membrane, monomer release into the ECM. Outside the cell, fibril assembly occurs with the resultant fibrils stabilised by cross-links. The following section describes events pertaining to the fibrillar collagens, unless stated otherwise, as little is yet known about non-fibrillar collagen biosynthesis.

In vertebrates, at least 30 separate collagen genes have thus far been described (Miller and Gay, 1987). The genes for the fibrillar collagens are large (52 exons, often 54 bp in length) and are derived from a single ancestor (Burgeson and Nimni, 1992; Chu and Prockop, 1993). In contrast, the genes for the non-fibrillar collagens show more diversity. The pro-α1(IV) gene has, for example, 52 exons as compared to the 47 exons of the pro-α2(IV) gene, while the pro-α1(X) gene contains only three exons (van der Rest *et al.,* 1993). Many of the non-fibrillar and fibrillar collagen gene transcripts have now been shown to undergo alternative splicing (Ryan and Sandell, 1990).

After the fibrillar gene is transcribed, it is spliced to yield a functional mRNA containing approx. 3000 bp (Figure 1.1a). The collagen mRNA is then translated and the nascent polypeptide chains are translocated across the rough endoplasmic

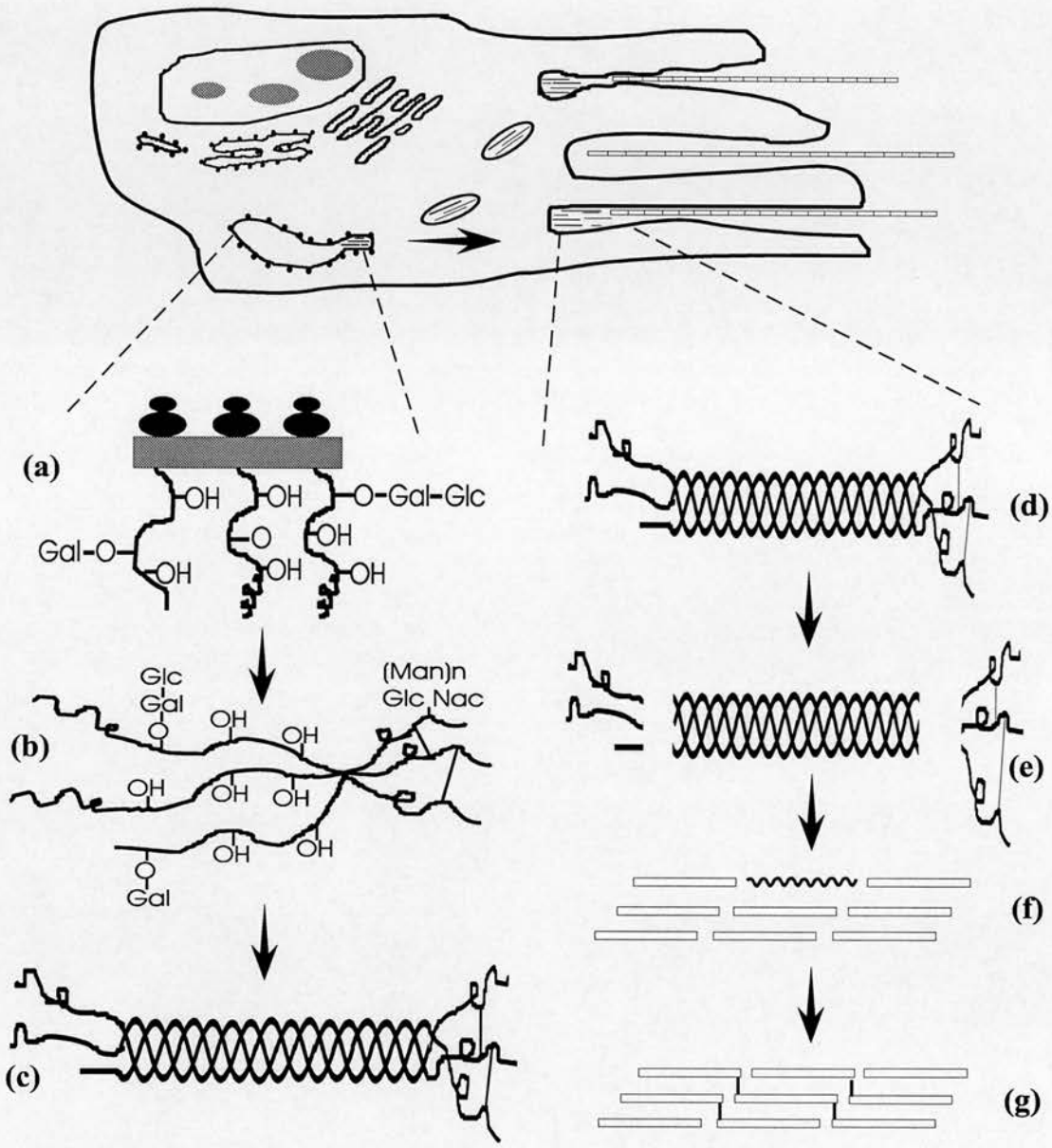


Figure 1.1 Production and assembly of collagen monomers into fibrils. The left-hand side of the diagram shows the events as they occur in the rough endoplasmic reticulum (rER): (a) translation, (b) post-translational modifications and (c) chain assembly. The right-hand side of the diagram shows extracellular events: (d) release of the procollagen into the extracellular space, (e) cleavage of the N- and C- propeptides, (f) fibril assembly and (g) cross-linking (adapted from Kadler, 1994).

reticulum (rER) to produce a soluble precursor (procollagen) comprised of 3 separate polypeptide chains (prepro-α chains).

Following cleavage of the signal peptides from the prepro-α chains within the rER, the resultant pro-α chains become subject to various post-translational modifications (Figure 1.1b). These include inter-chain disulphide cross-linking, hydroxylation of prolyl and lysyl residues, hydroxylysine O-linked glycosylation in the triple-helical region and N-linked glycosylation in the C-propeptide region (Hulmes, 1992; Kielty *et al.,* 1993; van der Rest *et al.,* 1993). The conversion of hydroxyproline in the Y position to 4-hydroxyproline is essential for the formation of hydrogen bonds and water bridges that aid stabilisation of the helix (Kuhn, 1987).

As these post-translational modifications take place, the collagen α-chains associate to form a central triple-helical collagen domain approximately 300nm in length and 1.5nm in diameter (Figure 1.1c; Hay, 1991). Each pro-α chain contains the repeating amino acid sequence Gly-Xaa-Yaa in its central region (Kadler *et al.,* 1996). The presence of glycine at every third residue of this sequence is essential: an amino acid with a bulkier side chain would be unable to occupy the centre of the triple helix that forms when the three α chains come together (Engel and Prockop, 1991). Proline often occupies the Xaa position of the repeating sequence, with hydroxyproline frequently being found at the Yaa site (Hulmes, 1992). Non-fibrillar collagens contain imperfections in the Gly-Xaa-Yaa sequence, for example Gly-Xaa-Gly-Xaa-Yaa or Gly-Xaa-Yaa-Xaa-Yaa, and it is these interruptions in the normal sequence that are responsible for the structural differences observed in the non-fibrillar collagens (Burgeson and Nimni, 1992).

Association of the C-terminal domains of the α-chains initiates trimer formation and assembly occurs in a zipper-like mechanism towards the N-terminus (Figure 1.1c; Engel and Prockop, 1991; Sandell *et al.,* 1991). The triple-helical domain is extended by propeptides both at the carboxyl and amino extremities, which are known as C- and N-propeptides respectively. The N-propeptide has been implicated in the feedback regulation of collagen synthesis and in the formation of collagen fibrils in the extracellular matrix (section 5.2; Brewton and Mayne, 1994).

Following transport through the golgi apparatus, procollagen monomers are aligned in register and packaged in secretory vesicles for release into the ECM (Figure 1.1d; Hulmes, 1992). In the extracellular matrix, specific enzymic cleavage of the non-helical propeptides by the procollagen N- and C-metalloproteinases, allows conversion of procollagen to collagen (Figure 1.1e; Kadler and Watson, 1995; Kadler *et al.,* 1995). Procollagen I C-proteinase (80 to 100kDa) is a neutral, Ca^{2+}-dependent single-polypeptide proteinase that is inhibited by metal chelators and also cleaves the C-propeptide from procollagens II and III (Hojima *et al.,* 1994b). It has recently been shown that C-proteinase is identical to bone morphogenetic protein-I (BMP-I; Kessler *et al.,* 1996).

Procollagen I N-proteinase (100 to 500 kDa), like procollagen C-proteinase, is a neutral, Ca^{2+}-dependent proteinase that is inhibited by metal chelators (section 4.1). This enzyme however, comprises up to 4 subunits and although it is also able to cleave procollagen II, it is not active on a procollagen III substrate (Tuderman and Prockop, 1982). These proteinases do not remove all non-helical regions as small telopeptides remain at both the N- and C-termini; these have been shown to be crucial in the assembly of fibrils (McPherson *et al.,* 1985).

The now insoluble, collagen monomers rearrange themselves and spontaneously self-assemble into fibrils that are staggered longitudinally with a 67 nm (D) periodicity (Figures 1.1f and 1.2; Eikenberry *et al.,* 1992). The assembly of collagen fibrils is an entropy driven process where the energy needed for association is derived from hydrophobic interactions (Kadler *et al.,* 1996). The role of the N- and C-propeptides in fibril formation remains unclear but it has been shown that both pN-collagen and pC-collagen are present at the fibril surface and it has been suggested that the N-propeptide may limit fibril diameter (section 1.3.4; Fleischmajer *et al.,* 1981).

Fibril formation is followed almost immediately by the formation of covalent cross-links both within and between collagen monomers (Figure 1.1g; Kielty *et al.,* 1993; Eyre *et al.,* 1990). These cross-links are formed from the action of lysyl oxidase on specific lysyl and hydroxylysyl residues and are responsible for conferring tensile

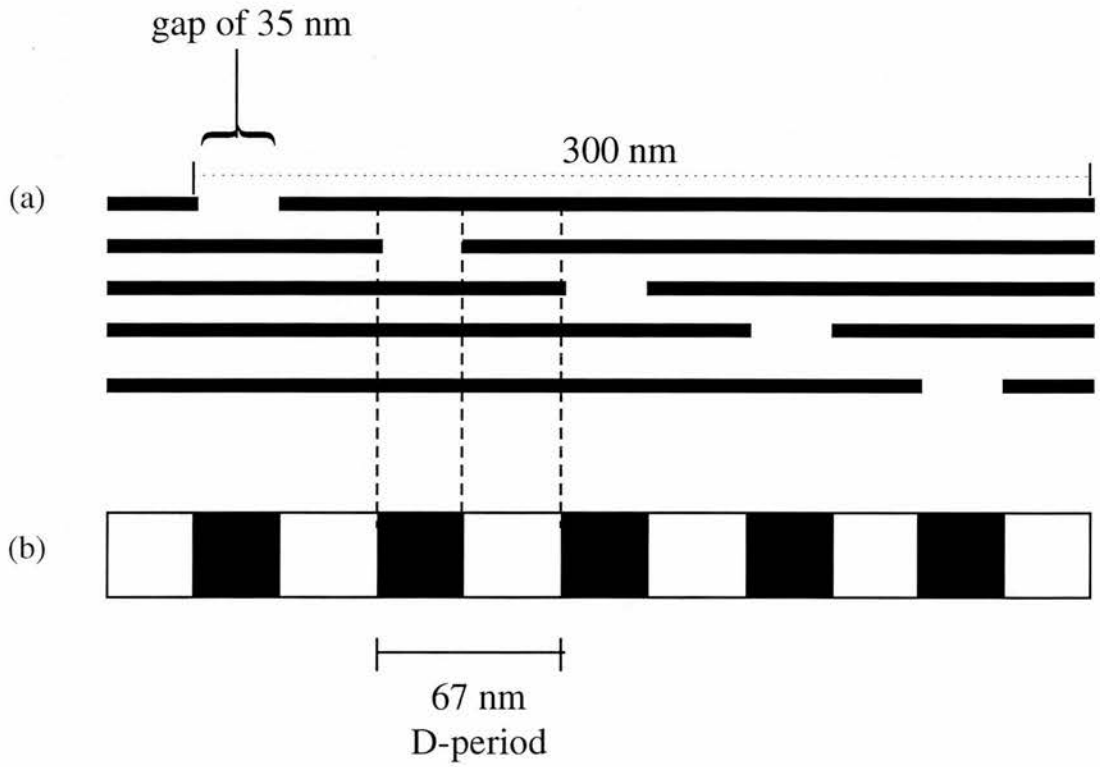


Figure 1.2 A collagen fibril (a) Schematic diagram illustrating the relationship between the staggered arrangement of collagen monomers within a fibril. (b) In negative staining, the stain permeates the fibrils, becomes trapped in the "hole" zones and thus appears as a dark band by TEM.

strength to the fibril (Kagan, 1986). Lysyl oxidase binds to the fibril surface implying that its action occurs at an early stage of fibrillogenesis (Cronland *et al.*, 1985).

1.2.3 Other ECM components

The other main constituents of connective tissues are the proteoglycans and glycoproteins (for reviews see Hardingham and Bayliss, 1990; Roughley and Lee, 1994; Iozzo and Murdoch, 1996). Proteoglycans are complex macromolecules that contain a core protein to which glycosaminoglycan side-chain(s) and N- and O-linked oligosaccharides are covalently attached (Wight *et al.*, 1991; Iozzo and Murdoch, 1996). Glycosaminoglycans (GAG's) are polysaccharide chains comprised of repeating disaccharide units that contain a derivative of an amino sugar (either D-glucosamine or D-galactosamine) and either hexuronic acid (D-glucuronic acid or L-iduronic acid) or galactose units. Sulphate groups are added to at least one of these sugar groups thus conveying a net negative charge to the molecule.

Proteoglycans can be divided into three categories based on the GAG chain that they contain: those with chondroitin sulphate (CS) or dermatan sulphate (DS) side chains, those with heparan sulphate (HS) side chains and those substituted with keratan sulphate (KS; Wight *et al.*, 1991). The same core protein may contain more than one type of GAG, while the number of side-chains attached to the core protein can vary from 1 to 100 (Roughley and Lee, 1994).

There are many different types of proteoglycans which display a variety of functions (Table 1.2). For example, decorin and fibromodulin interact with collagen fibrils to regulate fibrillogenesis and matrix organisation (Vogel *et al.*, 1984; Hedbom and Heinegard, 1993) while biglycan is found associated with the cell surface (Neame, 1993). The major proteoglycan of cartilage is aggrecan which shows a marked tendency to aggregate with hyaluronic acid (Aydelotte and Kuettner, 1993) and these proteoglycan aggregates allow cartilage to adapt to changing compressive loads (section 1.3.1).

NAME	*M_r (kDa)*	*SIDE CHAIN*	*FUNCTIONS*
Aggrecan	260	Chondroitin and keratan sulphate	The major proteoglycan of cartilage which endows it with compressive stiffness and resistance to deformation (Roughley and Lee, 1994).
Biglycan	40	Dermatan sulphate	Present pericellularly in cartilage but function unknown (Poole *et al.*, 1996)
Decorin	36	Dermatan sulphate	Widely distributed in tissues rich in collagens I and II where it interacts with collagen via its core protein rather than the GAG side-chain (Poole *et al.*, 1996). It has been shown to slow the rate of *in vitro* fibrillogenesis as well as to reduce the diameter of fibrils formed (Vogel *et al.*, 1984). The chick form has 2 dermatan sulphate side-chains (Blaschke *et al.*, 1996).
Fibromodulin	59	Keratan sulphate	Binds to collagens I and II and inhibits *in vitro* fibrillogenesis (Oldberg, 1989).
Hyaluronic Acid	300-2000	Not applicable	A glycosaminoglycan, not attached to a core protein. It is found associated with aggrecan and link protein in the form of large aggregates (Neame, 1993).
Versican	260	Chondroitin sulphate	May play a role in intracellular signalling or connecting ECM components to cell-surface glycoproteins (Ayad *et al.*, 1994).

Table 1.2. Hyaluronic acid and the major proteoglycans of cartilage.

Glycoproteins are a group of non-collagenous extracellular proteins, that includes fibronectin and laminin, which mediate cell adhesion, and tenascin, which modulates growth and differentiation during morphogenesis (Yamada, 1991; Fife and Brandt, 1993). One of the major glycoproteins present in cartilage is link protein which stabilises proteoglycan aggregates by binding both aggrecan and hyaluronic acid (Aydelotte and Kuettner, 1993). Other glycoproteins of cartilage include cartilage oligomeric matrix protein (COMP) which may function in adhesion of chondrocytes to each other or to other matrix proteins, and cartilage matrix protein (CMP), a 148kDa trimer that binds to collagen II in a D-periodic distribution although its function is unknown (Winterbottom *et al.,* 1992).

1.2.4 Tissues

Collagen molecules assemble to form complex patterns which determine the mechanical properties of various tissues and organs. For example, the multiple criss-crossing layers of collagen fibrils in the skin conveys multidirectional resistance to stretch, whereas the parallel bundles of fibrils in tendon are responsible for the high unidirectional tensile (Eikenberry *et al.,* 1992). Furthermore, the orthogonal arrangement of relatively narrow, uniform diameter collagen I fibrils in the cornea leads to optical transparency (Fitch *et al.,* 1994). The following section examines the structure of cartilage, a highly specialised connective tissue whose ability to withstand compressive and torsional forces throughout the body is due to its highly organised ECM.

1.3 CARTILAGE

There are three forms of cartilage that are distinguishable on the basis of their morphological and histological appearance as well as on their developmental history (Cancedda *et al.,* 1995). (1) Hyaline cartilage is a general term used to describe a range of connective tissues noted for their resilience and mechanical durability under various loading conditions (Poole, 1993). The most widely studied of the hyaline cartilages is articular cartilage (section 1.3.1). (2) Fibrous cartilage is an intermediate

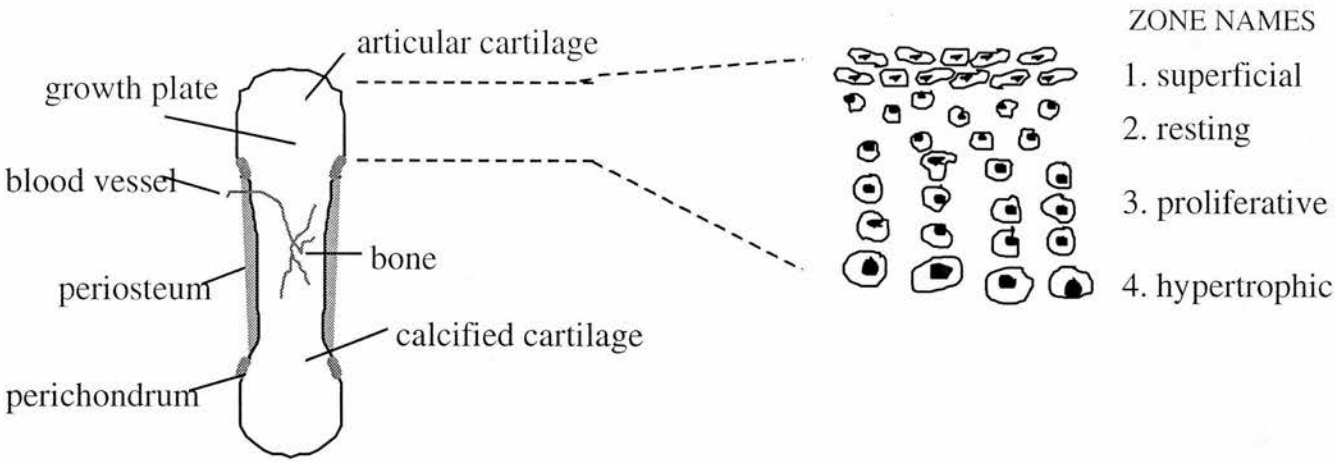
between fibrous tissue and hyaline cartilage. It is rich in collagen fibres that confer great resistance to traction and compression. Fibrous cartilage is found in the insertion zones of certain tendons and invertebral discs (Mallein-Gerin and van der Rest, 1996). (3) Elastic cartilage is characterised by the presence of elastic fibres and is the constituent of, for example, the epiglottis, the Eustachian tubes and certain cartilages of the larynx (Horton, 1993). This thesis is concerned with articular cartilage collagens and so the following section reviews current understanding of this tissue (for reviews see Kuettner *et al.,* 1990; Kuettner *et al.,* 1991; Horton, 1993).

1.3.1 Articular Cartilage Morphology

Articular cartilage is an aneural, avascular, alymphatic hyaline cartilage (Poole, 1993) that covers the ends of bones in synovial joints and provides smooth articulation, distribution of load, resistance to compressive forces and cushioning of the bone during joint movements (Kuettner *et al.,* 1991). These attributes are a direct consequence of its highly ordered ECM.

The main constituent of articular cartilage is water (65-80% tissue wet weight, Kuettner *et al.,* 1991), while collagens (50-60% dry weight) and proteoglycans (30-35% dry weight) make up most of the proteinaceous portion of the tissue (Buschmann *et al.,* 1992). Collagens form an extensive network of fibrils in which the other components are embedded (section 1.3.3; Eikenberry *et al.,* 1992) and all these ECM components are produced by cartilage-specific cells, called chondrocytes (section 1.3.2).

In cartilage, large proteoglycan aggregates (section 1.2.3) allow the tissue to adapt to changing compressive loads and hence to protect the underlying bone by retaining appropriate amounts of water. In unloaded joints, water is retained within the cartilage due to a swelling pressure exerted by the proteoglycans (Kuettner *et al.,* 1990). When cartilage is deformed, water is displaced which raises the negative charge density and increases the resistance to further deformation. When the load is



ZONE NAME	CHONDROCYTE APPEARANCE	I	II	III	VI	IX	X	XI
superficial	small, rounded and densely packed cells with little surrounding matrix.	✓✓✓	✓	✓✓	✓	✓	x	✓
resting	single, small cells surrounded by abundant matrix	x	✓✓	x	✓	✓✓	x	✓✓
proliferative	cells are in a column and are slightly larger	x	✓✓✓	x	✓✓	✓✓✓	x	✓✓✓
hypertrophic	cells have large cell bodies	x	✓✓✓	x	✓✓	✓✓	✓✓✓	✓✓✓

Figure 1.3. The growth plate of articular cartilage. The diagram shows the location of the growth plate with a magnified view showing the arrangement of the chondrocytes within this region. The table summarises the appearance of the chondrocytes and collagen expression in each zone (adapted from Wallis, 1996).

subsequently reduced, there is an influx of water into the tissue until the swelling pressure returns to normal (Maroudas *et al.,* 1986).

1.3.2 Chondrocytes

Chondrocytes are specialised cells whose main function is to maintain a fine balance between synthesis and breakdown of the ECM and thus regulate the integrity of cartilage (Muir, 1995). Chondrocytes are encapsulated within a specialised micro-environment termed a chondron (Poole *et al.,* 1985), a compression-resistant, fluid-filled "sack" that allows the cells to withstand high compressive loading *in vivo* (Poole, 1993; Urban, 1994; Muir, 1995; Lee *et al.*, 1997). Chondrocytes differ from most other cells in their lack of cell-to-cell contact (section 3.1) and, as compared with cells of other tissues (for example, skin), the proportion of chondrocytes in cartilage is comparatively low (2-10% tissue volume). This low concentration is likely to be due to limitations of nutrient and oxygen diffusion through the tissue (Stockwell, 1979).

In the growth plate, at the end of long bones, cartilaginous tissue is gradually replaced by bone during a process termed endochondral ossification (Cancedda *et al.,* 1995). Chondrocytes undergo various stages of differentiation during this process and pass sequentially through a proliferative, a hypertrophic and a degenerative stage (Castagnola *et al.,* 1986; Vornehm *et al.,* 1996). Based on these observations, articular cartilage can be classified into four zones: superficial, resting, proliferative and hypertrophic (Figure 1.3). In each zone, there are changes in collagen production, shifting from the synthesis of collagen I in the perichondral zone, to collagens II and IX in the proliferative region and to production of collagen X in the hypertrophic region (Castagnola *et al.,* 1988).

1.3.3 Cartilage Collagens

The collagen phenotype of articular cartilage is complex. Collagen II accounts for 90% of the collagen produced (Eyre *et al.,* 1991a; Mayne and Brewton, 1993) and it

interacts with collagens IX and XI to form a network of heterotypic fibres throughout the tissue (Vaughan *et al.,* 1988; Mendler *et al.,* 1989). Small amounts of collagens I, III, VI and X are also present but show restricted distributions and/or are more common in diseased tissues (sections 1.3.3.4 and 1.3.3.5). The cartilage collagens are now described in more detail.

1.3.3.1 Collagen II

Collagen II is a member of the fibrillar family of collagens and is a homotrimer comprising three α1(II) polypeptide chains, each containing 1050 amino acids (Ayad *et al.,* 1994). Collagen II differs from collagen I in that there is a higher hydroxylysine content and a higher rate of glycosylation (Kuhn, 1987). As described previously (section 1.2.2), it is synthesised in the form of procollagen monomers which, after the removal of the N- and C-propeptides, self-assemble into fibrils 10-40 nm in diameter, with a stagger of 234 amino acids (Figure 1.2; Ayad *et al.,* 1994; Fertala *et al.,* 1994).

The COL2A1 gene has recently been shown to undergo differential splicing of exon 2 to produce either procollagen IIA, which contains the cysteine-rich 69 amino acid domain of the N-propeptide, or procollagen IIB which does not (Figure 1.4; Ryan and Sandell, 1990). The latter is the predominant mRNA in cartilage and is expressed by cells that appear large and round, while procollagen IIA is expressed in cells which are "fibroblastic" in appearance (section 4.4.1; Sandell *et al.,* 1991). This indicates that the removal of the exon 2 encoded region from the pre-mRNA, and corresponding peptide sequence from the N-propeptide, may be an important step in cartilage development.

The diameter of collagen II fibrils in cartilage depends on the developmental stage and location within the tissue (section 1.3.4). In general, embryonic and growth plate cartilages are characterised by thin collagen fibrils of less than 25 nm diameter whereas mature articular cartilage usually contains thicker fibrils of 30-200 nm in diameter (Vaughan *et al.,* 1988; Mendler *et al.,* 1989; Keene *et al.,* 1995).

Overexpression of the α1(II) gene in transgenic mice, however, has been shown to produce abnormally thick fibrils, suggesting that an imbalance of the constituents of cartilage fibrils disrupts the mechanism that controls their assembly (Garofalo *et al.*, 1993). Also, the cartilage of COL2A1 knockout mice has been found to consist of highly disorganised chondrocytes that lack extracellular collagen fibrils, although many skeletal structures develop normally (Li *et al.*, 1995). Both these observations suggest that an organised ECM is essential for development of some components of the vertebrate skeleton, but it is not essential for others.

1.3.3.2 Collagen XI

Collagen XI is a member of the fibrillar collagen family and has been shown to form small diameter fibrils *in vitro* (Smith *et al.*, 1987). The molecule was originally isolated from human costosternal and femoral head cartilages and was called 1α2α3α collagen (Burgeson and Hollister, 1979). Chemical cross-linking showed that most molecules existed as heterotrimers and thus the three chains were renamed α1(XI), α2(XI) and α3(XI) (Eyre *et al.*, 1987b; Morris and Bachinger, 1987). The α3(XI) chain has now been found to be an over-glycosylated form of the α1(II) chain (Reese and Mayne, 1981; Bernard *et al.*, 1988) while the high sequence similarity of collagens V and XI indicates that these molecules should be regarded as isoforms (α1(XI)/α1(V) and α2(XI)/α2(V) are both 85% identical, α1(XI)/α2(XI) are 78% identical, α1(XI)/α3(V) are 70% identical while α1(XI)/α2(V) are less than 50% alike; Morris *et al.*, 1990; Petit *et al.*, 1993; Mayne *et al.*, 1993; Bruckner and van der Rest, 1994).

Collagen XI is also synthesised as a procollagen (Figure 1.1) and undergoes two proteolytic processing steps in its conversion to collagen. The first involves the loss of interchain disulphide bonds, thus implying the loss of the C-propeptide (Thom and Morris, 1991). The second step involves removal of the N-propeptide (Rousseau *et al.*, 1996), although there is retention of a pepsin-labile domain and it is likely that some molecules, whose N-terminal domains remain intact, are incorporated into a growing fibril (Morris and Bachinger, 1987).

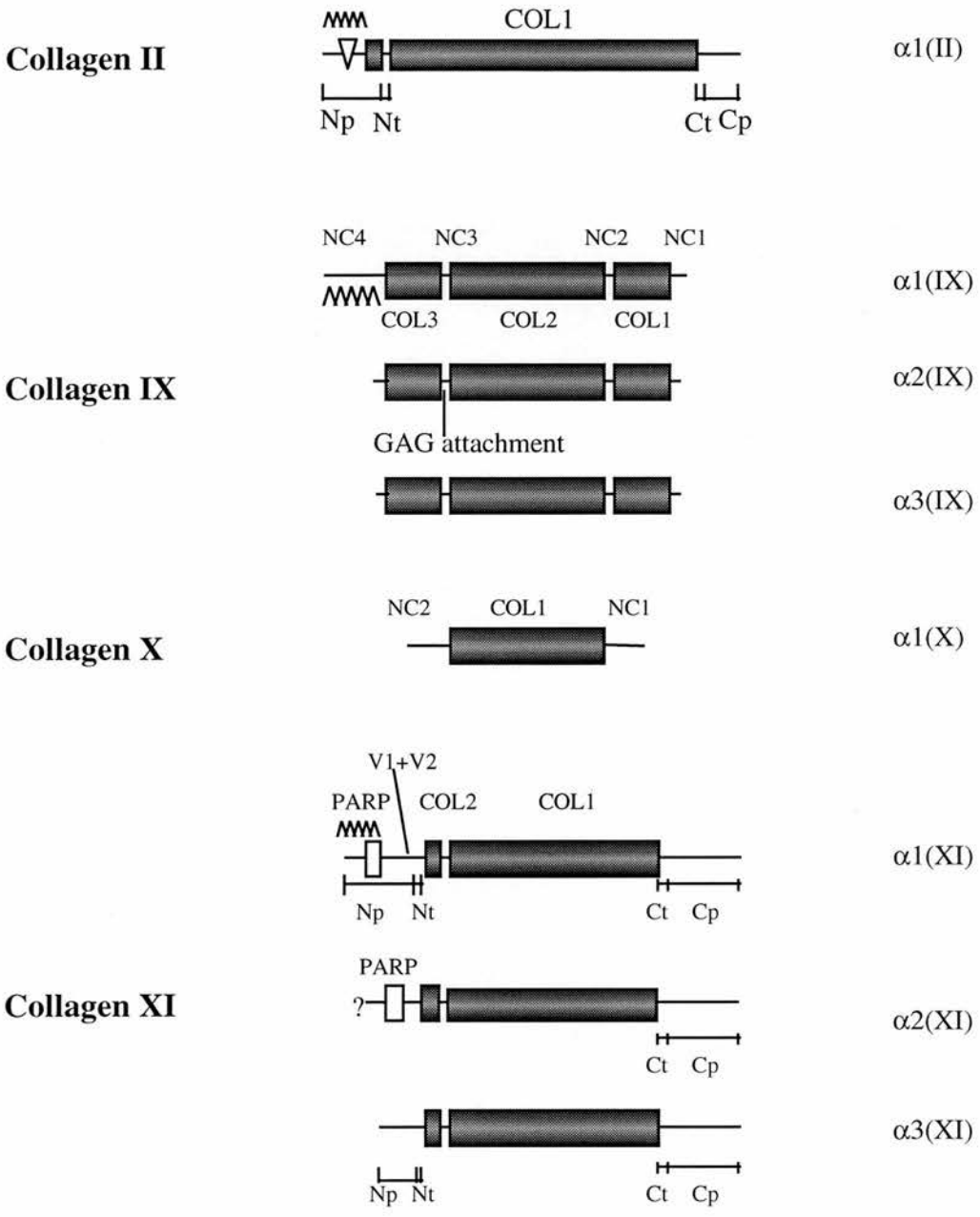


Figure 1.4 Diagram showing the different domains of each chain of collagens II, IX and XI. Np - N-propeptide; Nt- N-telopeptide; Ct - C-telopeptide; Cp - C-propeptide; COL - collagenous domain; NC - non-collagenous domain; PARP - proline-arginine rich protein; V1 and V2 - variable regions. ⩙ = alternatively spliced region; ∇ = propeptide; □ = PARP domain; ▮ = collagenous region; — = non-collagenous region.

The N-terminal domain of collagen XI is globular (>400 amino acids; Brewton and Mayne, 1994) and can be divided into a weakly basic N-terminal half containing cysteine residues (designated PARP) and a very acidic half adjacent to the minor helix that is known as the variable region (V1 and V2; Figure 1.4; Thom-Oxford *et al.,* 1995). The proline/arginine rich protein (PARP) domain was first isolated and characterised from bovine cartilage where it was shown to be part of the α1(XI) chain (Neame *et al.,* 1990). It has also been identified in human cartilage and demonstrated to be a fragment of the N-terminal domain of the α2(XI) chain (Zhidkova *et al.,* 1993) but its function remains unknown. The variable regions, V1 and V2, located between the PARP region and the minor helix, generate 6 forms of the N-terminal domain by alternative splicing (Thom-Oxford *et al.,* 1995). All six forms are present in cartilage but it is not known whether a single chondrocyte produces all six forms or whether the splice patterns correlate with the degree of chondrocyte differentiation. Since the V1 and V2 regions are initially retained on the surface of the fibril, the alternatively spliced regions could play a role in limitation of fibril diameter or influence interactions with other ECM molecules (Zhidkova *et al.,* 1995; Rousseau *et al.,* 1996).

Collagen XI was originally thought to be cartilage-specific (Mendler *et al.,* 1989) but evidence suggests that the α1(XI) chain associates with that of α2(V) (Niyibizi and Eyre, 1989) and this heterotypic molecule has now been identified in bovine bone and vitreous humour and the rhabdomyosarcoma A204 cell line (Mayne, 1989; Kleman *et al.,* 1992). Moreover, α1(XI) mRNA has been found in other non-cartilaginous embryonic tissues including brain, heart, skeletal muscle and skin (Nah *et al.,* 1992).

The function of collagen XI remains unclear although there is increasing speculation that, by analogy to collagens I and V, it plays a major role in limiting the diameter of collagen II fibrils (section 1.3.4; Eikenberry *et al.,* 1992). Collagen XI is cross-linked to collagen II (Wu and Eyre, 1995), but immunolabelling experiments show that the epitopes for the helical domain of collagen XI are unavailable in intact heterotypic fibrils (Petit *et al.,* 1993). Labelling occurs after disruption of these fibrils with

chaotropic agents, or by shearing, suggesting that collagen XI is at the core of the collagen II fibril (Mendler *et al.,* 1989). It is thought that from this location it limits the diameter of collagen II fibrils, which is supported by the finding that collagen XI is restricted to thin fibrils of bovine epiphyseal cartilage (Petit *et al.,* 1993; Keene *et al.,* 1995). Furthermore, in the *cho* mouse, where a mutation in the α1(XI) collagen gene limits the synthesis of the pro-α1(XI) chain, the animal displays an autosomal recessive chondrodysplasia characterised by abnormally thick cartilage fibrils (Seegmiller *et al.,* 1971; Seegmiller *et al.,* 1972; Li *et al.,* 1995). The mechanism by which collagen XI potentially limits the diameter of the collagen II fibril is unknown but again, by analogy to collagen V, it has been proposed that the flexible N-terminal region of collagen XI functions as the diameter regulator (section 1.3.4).

1.3.3.3 Collagen IX

Collagen IX is a member of the FACIT family of collagens (section 1.2.1) and accounts for 5-20% of total collagen in articular cartilage (McCormick *et al.,* 1987; Olsen, 1997). It is a heterotrimer composed of 3 genetically distinct α-chains, α1(IX), α2(IX) and α3(IX) which are present in equimolar amounts within a single molecule (van der Rest and Mayne, 1987).

Collagen IX has three triple-helical domains (COL1-3) interspersed by four non-collagenous domains (NC1-4) and is 200 nm in length (Figure 1.4; van der Rest *et al.,* 1985; Vaughan *et al.,* 1988). The COL1 and COL2 domains, separated by NC2, form the long "arm" of the molecule and are cross-linked to collagen II fibrils (Figure 1.5). COL3, which is separated from COL2 by the NC3 domain, forms the short "arm" and projects into the perifibrillar space. This COL3 domain has an unusually high imino acid content and thus increased helical stability, and its projection from the surface of the collagen II fibril potentially allows the NC4 domain to interact with other components of the matrix (Vasios *et al.,* 1988). The NC4 domain is derived largely from the amino terminus of the α1(IX) chain, with much smaller contributions from the α2(IX) and α3(IX) chains (Fassler *et al.,* 1994). It is

visualised as a "knob" by TEM (Brewton *et al.,* 1991) and has a pI of 9.8 making it an ideal candidate for the interaction with polyanionic proteoglycans that are abundant in cartilage (Vasios *et al.,* 1988). The vitreous humour form of collagen IX does not contain a NC4 domain; these different isoforms arise from use of two different promoters during transcription of the α1(IX) gene (Svoboda *et al.,* 1988; Yada *et al.,* 1990; Brewton *et al.,* 1991). The upstream promoter gives rise to the cartilage form while the downstream promoter leads to the vitreous form.

Collagen IX can also exist as a proteoglycan when it has a single chondroitin sulphate glycosaminoglycan (GAG) chain attached to the α2(IX) polypeptide chain (Huber *et al.,* 1986; Nishimura *et al.*, 1989). The GAG chain is attached to the serine residue of the peptide sequence Gly-Ser-Ala-Asp located in the NC3 domain (McCormick *et al.,* 1987) which is also the point at where a kink in the molecule is observed by TEM (Reese *et al.,* 1982). Pepsin treatment creates a gap in the NC3 domain of α2(IX) and leads to the loss of the GAG chain (van der Rest and Mayne, 1987). The role of the GAG chain is unclear, but it has been proposed that its presence may guide collagen IX to the correct docking site on the surface of the collagen II fibril, after which it is cleaved to generate the final, non-proteoglycan form of the molecule (Diab *et al.,* 1996).

There are several interchain disulphide bonds in collagen IX that are formed from the cysteine residues found in the NC1 and NC3 domains (van der Rest *et al.,* 1985; van der Rest and Mayne, 1988). There are also at least two sites for the cross-linking of collagens II and IX, one of which involves the formation of a hydroxypyridinium cross-link between the COL2 domains of the α2(IX) and α3(IX) chains and the telopeptides of two α1(II) chains (Figure 1.5; Eyre *et al.,* 1987a; van der Rest and Mayne, 1988; Shimokomaki *et al.,* 1990; Wu *et al.,* 1992).

There has been considerable speculation about the function of collagen IX, much of which revolves around the hypothesis that collagen IX acts to stabilise the collagen network. Some of the potential functions are listed below:

- Collagen IX maintains spacing of the collagen network. The localisation of collagen IX to the surface of collagen II fibrils would allow the highly cationic

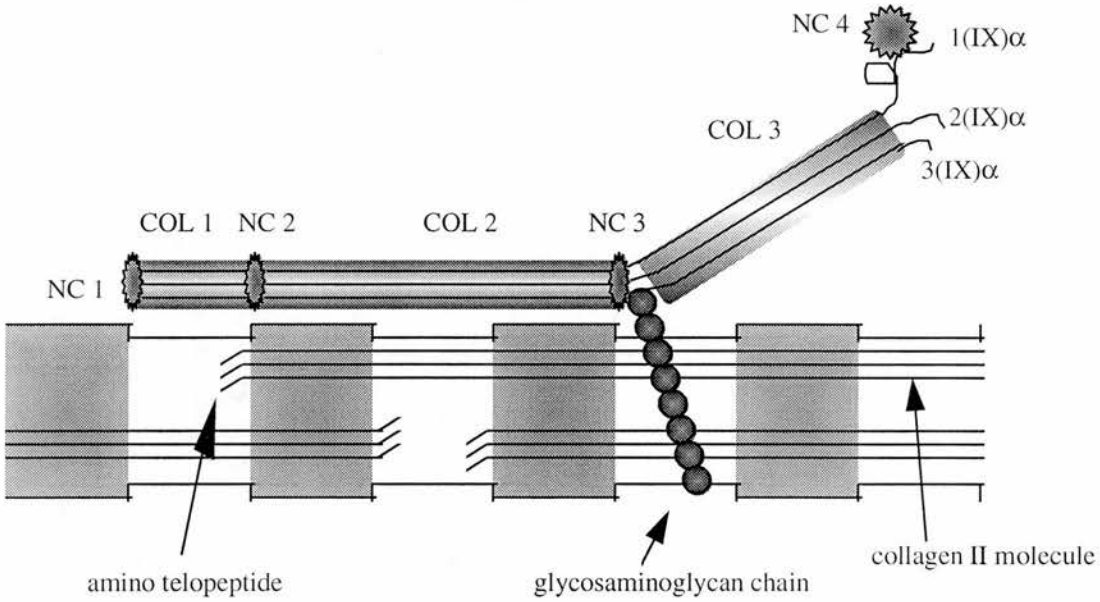


Figure 1.5. Collagen IX is cross-linked to the surface of collagen II-containing cartilage fibrils. Collagen IX consists of 3 genetically distinct chains, which form a long (NC1, COL1, NC2, COL2) and a short "arm" (COL3) and are connected by a flexible hinge region (NC3). There is a large disulphide-bonded globule domain (NC4) located at the N-terminus of COL3 and a glycosaminoglycan chain is attached to the α2(IX) chain in the NC3 region. Collagen IX molecules are covalently cross-linked to collagen II (adapted from Nishimura *et al.*, 1989).

- NC4 domain to interact with the polyanionic GAG chains of interfibrillar proteoglycan aggregates (Figure 1.5; van der Rest and Mayne, 1988; Smith and Brandt, 1992). Adjacent collagen II fibrils could then be "glued" together if the NC4 domains of several collagen IX molecules interacted with a common proteoglycan aggregate. This would stop collagen II fibril-fibril interactions and thus maintain fibril spacing within the network (Wotton *et al.*, 1988).

- Collagen IX limits the diameter of heterotypic collagen fibrils. Collagen IX was initially located to intersections of collagen II fibrils in chick embryonic sternal cartilage (Muller-Glauser *et al.,* 1986), but has since been shown to decorate the surface of collagen II fibrils in a D-periodic distribution (Vaughan *et al.,* 1988). This distribution has led to the suggestion that it may have a role in the limitation of lateral growth of these fibrils (section 1.3.4; Duance *et al.,* 1990; Wu *et al.*, 1992) and that its turnover may be integral to growth and remodelling of the fibrillar matrix (Lefkoe *et al.*, 1997).

- Collagen IX may act as a "fusible link" that can be cleaved by mechanical forces or by proteases. For example, stromelysin-1 cleaves in NC2 which removes the NC4 domain and this action may provide either a specific mechanism for reorganisation of the collagen network (Wu *et al.,* 1991), or may act to initiate the swelling and lost cohesiveness characteristic of osteoarthritic cartilage (section 1.3.5; Muir, 1990).

- Collagen IX may play a role in the regulation of compressive forces, as its distribution along collagen II fibrils may shield the fibril core from proteoglycan aggregates and thereby establish a low-density, water-rich layer around the fibrils. This would reduce friction between fibrillar and non-fibrillar components during compression/decompression (Studer *et al.,* 1996).

1.3.3.4 Collagen X

Collagen X is a short-chain collagen that serves as a highly specific marker for chondrocyte maturation (O'Keefe *et al.*, 1994). The collagen X molecule is a

homotrimer of three α1(X) chains and consists of three protein domains; a short triple helix (COL1) is flanked by the non-helical amino-terminal (NC2) and carboxyl-terminal (NC1) domains (Figure 1.4; Chan *et al.*, 1996). This collagen type is mainly found in cartilage but also occurs at low levels in intramembranous bone. Collagen X can assemble into fine pericellular filaments found in association with collagen II fibrils or into hexagonal lattices (Schmid and Linsenmayer, 1990; Kwan *et al.*, 1991).

In cartilage, collagen X is only synthesised by hypertrophic chondrocytes and therefore it may play an important role in the processes of calcification or chondrocyte maturation (Wardale and Duance, 1993). Alternatively, collagen X may facilitate degradation and removal of hypertrophic matrix as there are two collagenase cleavage sites on the molecule (Schmid and Linsenmayer, 1990).

1.3.3.5 Other Collagens Present in Cartilage

Collagens I, III and VI are also detected in cartilage (Figure 1.3), although they comprise a relatively small proportion of the total collagen present. Collagen I is found in very small quantities in the superficial zone of adult human cartilage and is a product of the flattened chondrocytes found in this area (Aigner *et al.*, 1993). Collagen III has been identified in osteoarthritic cartilage and also in small amounts in normal human articular cartilage but its function remains unclear (Wotton and Duance, 1994). Collagen VI is present in bovine articular cartilage and represents ~1-2% of the total collagen (Eyre *et al.*, 1987b). This collagen type is found associated mainly with chondrocytes in the deeper zones of cartilage where it is an integral part of the chondron, although it has also been located in the superficial zone where it interacts strongly with collagen I (Ayad *et al.*, 1989; Bonaldo *et al.*, 1990). Collagen VI is thought to have a role in cell attachment due to its pericellular distribution and the fact that it contains RGD sequences (Wardale and Duance, 1993).

1.3.4 Collagen Fibril Assembly

One noticeable feature of cartilage is that the collagen fibrils vary in diameter depending on both developmental stage and location within the tissue (section 1.3.3.1; Eikenberry *et al.,* 1992). It is intriguing that fibrils reconstituted *in vitro* from purified collagen II show no control of fibril width although the fibrils exhibit identical banding patterns to those found *in vivo* (Lee and Piez, 1983). Therefore the question arises as to what controls the width of cartilage collagen fibrils *in vivo*.

Recently, fibrils *in vivo* have been found to be comprised of more than one collagen type, for example, collagens I and V in the cornea, collagens I and III in skin and collagens II, IX and XI in cartilage (Birk *et al.,* 1988; Fleischmajer *et al.,* 1990; Mendler *et al.,* 1989). It was therefore suggested that control of fibril diameter was due to interactions between these different collagen types, which has now been directly demonstrated for collagens I and V in the cornea. When collagen I was reconstituted in the presence of increasing amounts of collagen V, there was a concomitant decrease in fibril diameter (Adachi and Hayashi, 1986; Birk *et al.,* 1990). Moreover, the observations that (1) antibodies to the triple-helical domain of collagen V did not label heterotypic fibrils (Linsenmayer *et al.*, 1993; Fitch *et al.,* 1994), and (2) the removal of the N-terminal domain of collagen V resulted in loss of diameter regulation (Linsenmayer *et al.,* 1993), led to the proposal that collagen V forms an inner core fibril whose N-terminal domains project out to the surface of the collagen I fibril, thus limiting its diameter. More recent evidence has shown that a decrease in the level of collagen V secreted by chick corneal fibroblasts *in vivo* led to an increase in the diameter of collagen I fibrils (Marchant *et al.,* 1996). Cartilage fibrils are also heterotypic and, by analogy to the mechanism by which collagen V regulates the diameter of collagen I fibrils in the cornea, it has been suggested that collagen XI regulates the diameter of collagen II fibrils in cartilage. This proposal is substantiated by the fact that collagens V and XI show high amino acid sequence similarities (section 1.3.3.2).

There have, however been other proposals that pertain to the control of fibril width *in vivo*. For example, the interaction of collagen fibrils with other ECM components has

been found to limit their lateral growth (MacBeath *et al.,* 1993; Kuhn, 1987). The proteoglycans, decorin and fibromodulin partially control the diameter of *in vitro* reconstituted collagen II fibrils (Vogel *et al.,* 1984; Hedbom and Heinegard, 1989). Moreover, the collagen fibrils of decorin knockout mice are loosely packed and vary in diameter along their length (Danielson *et al.,* 1997). The localisation of collagen IX, that also occurs as a proteoglycan *in vivo*, to the surface of cartilage fibrils suggests a role for this collagen type in fibril diameter regulation (section 1.3.3.3; Duance *et al.*, 1990; Wu *et al.,* 1992).

The propeptides of procollagen have also been suggested to play a role in fibril diameter regulation as both the N- and C-propeptides have been localised either on or near collagen fibrils (Fleischmajer *et al.*, 1981). Moreover, changes in fibril diameter are accompanied by changes in the pN/pC-collagen ratio (Fleischmajer *et al.,* 1985). It has also been proposed that the selective removal of the N-propeptide from the pro-α1(XI) chain at the surface of a growing fibril is a limiting step in the assembly of heterotypic cartilage fibrils (Miyahara *et al.,* 1984; Chapman, 1989; Thom and Morris, 1991).

Cells are also believed to play a role in controlling lateral growth of collagen fibrils. The initial events of fibril assembly, for example cleavage of propeptides and assembly of early fibrils, are under tight cellular control (Birk *et al.,* 1989). Moreover, the finding that segment-long-spacing (SLS) crystallites are observed within vacuoles of fibroblasts indicates that the initial stages of fibril assembly may occur intracellularly (Bruns *et al.,* 1979). Alternatively, cells could add components to block the fibril assembly process after a certain diameter has been attained (Eikenberry *et al.,* 1992).

Other explanations for the control of fibril diameter also exist. For example, as the fibril diameter increases, addition of further molecules to the fibril becomes energetically unfavourable and thus fibrils tend to remain thin (Wood, 1960). Alternatively, the large number of carbohydrate moieties on collagen II monomers affect fibril diameter (Kuhn, 1987). It is likely that a combination of the aforementioned proposals leads to the precise control of fibril diameter that is

observed *in vivo*, and that the different mechanisms exist to provide a "back-up" system in case of failure of one or more of the mechanisms.

1.3.5 Diseases of Articular Cartilage

Many diseases of cartilage have been characterised which mostly affect collagen II genes (Mayne, 1989). For example, recent evidence has suggested that mutations in the COL2A1 gene that affect the triple helical domain of collagen II, delay triple-helix formation resulting in post-translational overmodification of the collagen (Chen *et al.,* 1996). Mutations in COL2A1 have been shown to cause severe dysplasias and osteoarthritis in both mice and humans (Olsen, 1995).

The chondrodysplasias are a group of diseases characterised by shortened limbs and skeletal deformities (Table 1.3; Horton and Hecht, 1993). Kneist dysplasia is one such chondrodysplasia and analysis of the cartilage in this condition has demonstrated several small deletions in the COL2A1 gene:

- a 28 bp deletion in the exon 12/intron 12 boundary results in skipping of exon 12 (Winterpacht *et al.,* 1993)
- a point mutation in intron 20 causes a deletion of 6 amino acids in exon 21 (Winterpacht, 1994)
- a point mutation in exon 12 results in alternative splicing of α1(II) pre-mRNA and thus deletion of 7 amino acids (Chen *et al.,* 1996)
- a 18 bp deletion in exon 49 results in the removal of two Gly-Pro-Pro triplets that are essential in the folding of the triple helix (Winterpacht *et al.,* 1996).

Stickler syndrome is another chondrodysplasia that affects the eyes, ears and skeleton (Table 1.3; Horton and Hecht, 1993). It been shown to be caused by a mutation that encodes a premature stop codon in the C-propeptide region of procollagen type II (Ahmad *et al.,* 1995). Mutations of the COL2A1 gene also affect collagen XI chains, as two families with an inherited chondrodysplasia resembling that of Stickler syndrome harbour an 18 amino acid deletion in the triple-helical domain of the α2(XI) collagen chain (Li *et al.,* 1995; Olsen, 1995; Winterpacht *et al.,* 1996).

DISORDER	*CLINICAL PHENOTYPE*	*PATHOLOGICAL PHENOTYPE*	*BIOCHEMICAL FEATURES*	*REF.*
Achondroplasia	short-limbed dwarfism, bow legs	short growth plate	defective regulation of chondrocyte proliferation or differentiation	Horton and Hecht, 1993
Diastrophic dysplasia	short-limbed dwarfism, club foot, cleft palate	thickening of collagen II fibrils, appearance of SLS, degenerating cells	unknown	Wallis, 1995
Kneist dysplasia	shortened trunk and limbs	soft cartilage with lesions; collagen fibrils are abnormally thin; retention of collagen II C-propeptide within the chondrocyte.	small deletions in COL2A1	Poole *et al.*, 1988
Stickler syndrome	cleft palate, severe myopia, degenerative arthritis	abnormal articular cartilage but not due to abnormal collagen II at the protein level	nonsense mutations in COL2A1 leading to a premature stop codon	Ahmad *et al.*, 1995
Thanatophoric dysplasia	short limbs, long narrow trunk, etc.	lack of chondrocyte proliferation and differentiation to hypertrophic chondrocytes	defect in COL2A1?	Horton and Hecht, 1993

Table 1.3 Chondrodysplasias. Some of the clinical and biochemical features associated with selective chondrodysplasias.

Mutations in the COL2A1 gene have also been found to be responsible for the appearance of osteoarthritis in some patients (Eyre *et al.*, 1991b; Ala-kokko *et al.*, 1990; Lefkoe *et al.*, 1997; Matyas *et al.*, 1997). Osteoarthritis (OA) is a common disease that produces joint pain and stiffness together with a progressive degeneration of joint cartilage (Grushko *et al.*, 1989). The earliest feature of osteoarthritic cartilage is an increase in water content that leads to a swelling of the collagen network (Diab *et al.*, 1996). Another early event in osteoarthritis is proteoglycan depletion (Curtin and Reville, 1995) although there is increased production of collagen II. The ECM, however is not restored to its former state as the now-damaged chondrocytes produce proteases that cause the irreversible breakdown of the collagen network (Sandy *et al.*, 1984; Aydelotte *et al.*, 1986).

Collagen IX is thought to be important in OA as some patients exhibit a decrease or absence of collagen IX in their articular cartilage (Brierly *et al.*, 1991). It should also be noted that a decreasing amount of collagen IX in the elderly correlates to a higher incidence of OA (Brierly *et al.*, 1991). Collagen IX has been suggested to have a role in the stabilisation of the fibrillar network (section 1.3.3.3) so its depletion by proteases could weaken the collagenous network and allow the tissue to swell. Also, transgenic mice with a dominant negative mutation in the α1(IX) chain develop degenerative joint disease (Nakata *et al.*, 1993; Fassler *et al.*, 1994).

Another point of interest is that there is an alteration in the expression of collagen phenotype in OA; collagens I, III and X are synthesised in all zones of articular cartilage (Adam and Deyl, 1983) indicating that this disorder results from an imbalance in the influence of factors controlling chondrogenesis and terminal chondrocyte differentiation respectively (Tschan *et al.*, 1993).

1.4 *In Vitro* Models of Cartilage Deposition

In vivo studies of cartilage are difficult. The effects of, for example, growth factors on chondrocyte differentiation are impossible to assess as other molecules may also be affected which could in turn influence differentiation. Moreover, studies on

normal human cartilage are difficult as this tissue is not readily available. These obstacles have partially been overcome by the development of *in vitro* systems which can analyse the effects of a single parameter change.

The role of a certain protein in the ECM can be directly assessed by use of transgenic mice. These have been used for a number of years to investigate the phenotypic changes brought about when a gene of interest is disrupted. For example, transgenic mice with a dominant negative mutation in the α1(IX) chain develop degenerative joint disease indicating the importance of this collagen type in cartilage (Nakata *et al.,* 1993; Fassler *et al.*, 1994). Also, the skin of decorin knockout mice exhibits loosely packed collagen fibrils which vary in diameter compared to the tightly packed, strictly controlled diameter fibrils characteristic of normal skin (Danielson *et al.,* 1997). Conversely, collagen X null mice show no skeletal deformities and grow normally indicating a non-essential function of this protein in the ECM (Chan *et al.*, 1996).

Cell culture systems have been introduced to study chondrocyte differentiation and ECM production *in vitro* (Cancedda *et al.*, 1995). To reduce problems of chondrocyte dedifferentiation, suspension cultures are used to maintain chondrocyte phenotype (section 2.1). The effects of lymphokines, growth factors and other parameters on chondrocyte growth rate, morphology and the type and amount of matrix molecules synthesised can easily be assessed (Tschan *et al.*, 1993; Bohme *et al.*, 1995). Thus, these culture systems have become a powerful tool for studying the mechanisms involved in cartilage induction and development. Moreover, a relatively small amount of starting material is required and as the cells can be passaged many times, this overcomes the lack of availability of certain tissues.

Alginate bead cultures are widely used as a suspension culture system for chondrocytes. This thesis addresses the question of "*Do alginate beads provide an environment similar to that of cartilage for the culture of chick chondrocytes*?". Biochemical analyses show that cartilage-specific collagens are produced in ratios similar to those found *in vivo* while sulphated glycosaminoglycans are retained in the cell-associated matrix over a 14 day culture period, suggesting proteoglycan

aggregation (Chapter 2). There were, however, 2 major differences between the ECM produced in alginate beads and that of cartilage: morphological analysis found that collagen did not form the expected cross-banded fibrils characteristic of cartilage while a higher amount of pN-collagen II (a precursor of collagen II) was produced in alginate culture. Chapter 3 therefore investigates the morphology of the chondrocytes and the ECM in alginate bead culture while chapter 4 examines methods of removal of the pN-collagen II. The final discussion considers whether this culture system provides a useful model for the study of *in vitro* cartilage deposition. Materials and methods are included in each research chapter, while protocols for the more general methods can be found in Appendix I.

CHAPTER 2

ALGINATE BEAD CULTURE OF EMBRYONIC CHICK CHONDROCYTES

2.1 INTRODUCTION

A number of *in vitro* models have been developed to investigate chondrocyte differentiation, though these studies have been complicated by the fact that chondrocytes are renowned for not maintaining their phenotype in culture (Cancedda *et al.*, 1995). Chick chondrocytes in monolayer culture soon start to dedifferentiate and fail to undergo cell division after the fifth passage (Holtzer *et al.*, 1960; Horwitz and Dorfman, 1970). The cells become fibroblast-like producing collagens I and III and not collagens II, IX and XI as would be expected (Eikenberry *et al.*, 1992). It was found however that when chondrocytes from these cultures were placed in suspension, their programme of collagen expression changed and collagens II, IX and XI were re-expressed (Benya and Schaffer, 1982; Tschan *et al.*, 1993). Suspension cultures thus became the preferred method of chondrocyte culture, as minimising cell-substratum interactions seems to maintain the chondrocyte phenotype. Unlike most other cells, except tumour and transformed cells, chondrocytes can proliferate and are metabolically active when grown in three-dimensional culture systems (Cancedda *et al.*, 1995).

The first material used for suspension cultures was soft agar. Dedifferentiation did not occur and approximately 30-35 doublings were observed before senesence occurred (Horwitz and Dorfman, 1970). Later low-temperature agarose was introduced as a support matrix (Benya and Schaffer, 1982; Bounelis and Daniel, 1983; Thomson *et al.*, 1985; Delbruck *et al.*, 1986). Chondrocytes were shown to assemble a functional ECM composed of proteoglycans and heterotypic collagen fibrils that displayed a characteristically uniform diameter of ~20 nm, similar to that of cartilage *in vivo* (Bruckner *et al.*, 1989).

More recently, Guo *et al.* (1989) developed the use of alginate beads as an alternative for chondrocyte culture. Alginate is a negatively charged polymer that, in the presence of calcium, polymerises to form a gel. Unlike agarose it has the advantage that in the presence of a calcium chelator (for example, 55mM sodium citrate or 50mM EDTA) the alginate readily dissociates, so allowing rapid recovery and quantitation of the chondrocytes and ECM proteins. Following dissociation and mild

centrifugation, two compartments are recovered: the chondrocytes and their associated matrix (CM) and the further removed matrix (FRM; Hauselmann *et al.*, 1992; Mok *et al.*, 1994). These compartments represent the combined pericellular and territorial matrices and the interterritorial matrix respectively (Figure 3.1). Recent studies on adult bovine chondrocytes cultured in alginate beads showed that these cells remained phenotypically stable for up to 8 months (Hauselmann *et al.*, 1994) and that 90% of total collagen is recovered from the CM and 10% from the FRM (Petit *et al.*, 1996).

Two previous studies have focused on collagen production in alginate beads, using chondrocytes obtained from rabbit or bovine sources (Ramdi *et al.*, 1993; Petit *et al.*, 1996), but little information is available on the alginate bead culture of embryonic chick chondrocytes. Due to the differences, both biochemically and morphologically, of the ECM's of different species (Elima and Vuorio, 1989), it was of interest to determine the identity of the collagens produced by chick chondrocytes cultured in alginate beads. Also, as cell culture is an important technique for the study of cartilage ECM *in vitro* (section 1.4) it becomes essential to know whether alginate beads provide an environment like that of cartilage for the culture of chondrocytes. This chapter analyses the biochemical features of the ECM produced by chick chondrocytes in alginate beads, which includes investigation of the collagen types produced and their ratios, determining whether collagen IX is present as a proteoglycan and/or a non-proteoglycan form and examining proteoglycan production. These features are compared directly to those of native articular cartilage.

The cross-link inhibitor β-aminopropionitrile (βAPN) is traditionally added to cell culture systems where collagens are produced as it allows easier recovery of these proteins (section 2.3.2), but nothing is known about its effect on chondrocytes or ECM production. Cell numbers, the distribution of collagens in each compartment and ECM production were analysed after the addition of 0.2mM βAPN to the culture medium, and the results were compared to cultures in the absence of βAPN.

2.2 MATERIALS AND METHODS

2.2.1 Materials

Fertile eggs were obtained from Ross Breeders, Newbridge, Midlothian; DMEM, trypsin, penicillin-streptomycin, fungizone, FBS and PBS were all purchased from Gibco Life Technologies, Paisley; Bacterial collagenase was from Boehringer, Lewes, East Sussex; Alginic acid (medium viscosity), pepsin, βAPN (fumarate salt), PMSF, NEM, EDTA, chondroitinase ABC, chondroitin sulphate (from shark cartilage), high molecular weight markers (cross-linked phosphorylase b), papain and CHAPS were from Sigma Chemicals Co., Poole, Dorset; [5-^{3}H]proline and "Amplify" were from Amersham, Aylesbury, Bucks.; Biomax film was from Kodak, Cambridge; DEAE-Sephacel was from Pharmacia/LKB, Milton Keynes; polyclonal antibodies to collagens II and IX and collagen X were kind gifts from Dr Janet Anderson-McKenzie and Dr Erik Hedbom, Munster, Germany, respectively. All other chemicals (analytical grade) were from BDH, Poole, Dorset, unless stated otherwise.

2.2.2 Alginate Bead Culture

Chondrocytes were released from 20 dozen, 17 day old chick embryo sterna using 12.5mg/ml trypsin and 22mg/ml bacterial collagenase in Dulbecco's Modified Eagles Medium (DMEM) containing 1% (v/v) penicillin-streptomycin (PS) and 1% (v/v) fungizone, at 37 ^{o}C. After digestion was nearly complete (approx. 2 hours), the cell suspension was filtered through a double layer of lens tissue and washed twice with an equal volume of DMEM containing 10% (v/v) fetal bovine serum (FBS), 1% PS and 1% fungizone. The cells were collected by centrifugation at 300g for 6 mins and resuspended in phosphate buffered saline (PBS).

The chondrocytes were subsequently washed twice in PBS to remove traces of FBS and suspended in alginate beads (Figure 2.1; Guo *et al.,* 1989). Briefly, a sterile 2% (w/v) alginate solution in 0.3M NaCl was diluted 1:1 with the cell suspension and drawn into a 21 gauge needle. The cell suspension (final concentration $2x10^{6}$

cells/ml, as determined by a haemocytometer) was extruded slowly through the needle into a solution containing 100mM $CaCl_2.2H_2O$ in 0.15M NaCl and the beads formed spontaneously. The beads were allowed to polymerise in this solution for 10 mins before being transferred to DMEM containing 10% FBS, 1% PS and 1% fungizone, supplemented with 0.28mM L-ascorbic acid and in the presence or absence of 0.2mM β-aminopropionitrile (βAPN). The culture flasks were maintained at 37 °C in a humidified atmosphere of 5% CO_2, and newly synthesised collagens were labelled with 5 μCi/ml [5-^{3}H]proline. The medium was changed daily for up to 14 days.

On each day stated, the medium was collected and the alginate beads solubilised with 55mM sodium citrate in 0.15M NaCl at 37 °C (1:5 alginate:citrate). The chondrocytes were collected by centrifugation (300g for 6 mins) and re-suspended in extraction buffer (1M NaCl, 50mM Tris-HCl, pH 7.5), in the presence of proteinase inhibitors (1mM PMSF, 10mM NEM and 2mM EDTA). Three fractions were therefore collected: medium, solubilised alginate (hereafter referred to as further-removed matrix; FRM) and the cells with their associated matrix (CM).

2.2.3 Analysis of Crude Extract

Collagens present in the CM extract were dialysed against 0.5M acetic acid and digested with pepsin (100 μg/ml) overnight at 4 °C with constant agitation (section 2.2.5). After neutralising each sample with 5M sodium hydroxide (NaOH), proteins were precipitated by the addition of 3 volumes of 95% (v/v) ethanol containing 1% (w/v) potassium acetate at 0 °C and collected by centrifugation (9500g for 5 mins). Proteins present in this crude extract were separated by SDS-PAGE with 6% separating and 4.5% stacking gels (reducing conditions). The gels were examined by fluorography and western blotting with polyclonal antibodies against collagens II, IX or X (see Appendix I for full details of both procedures). The primary antibody dilution was 1:3000 and the secondary antibody (goat anti-rabbit) dilution was 1:2000.

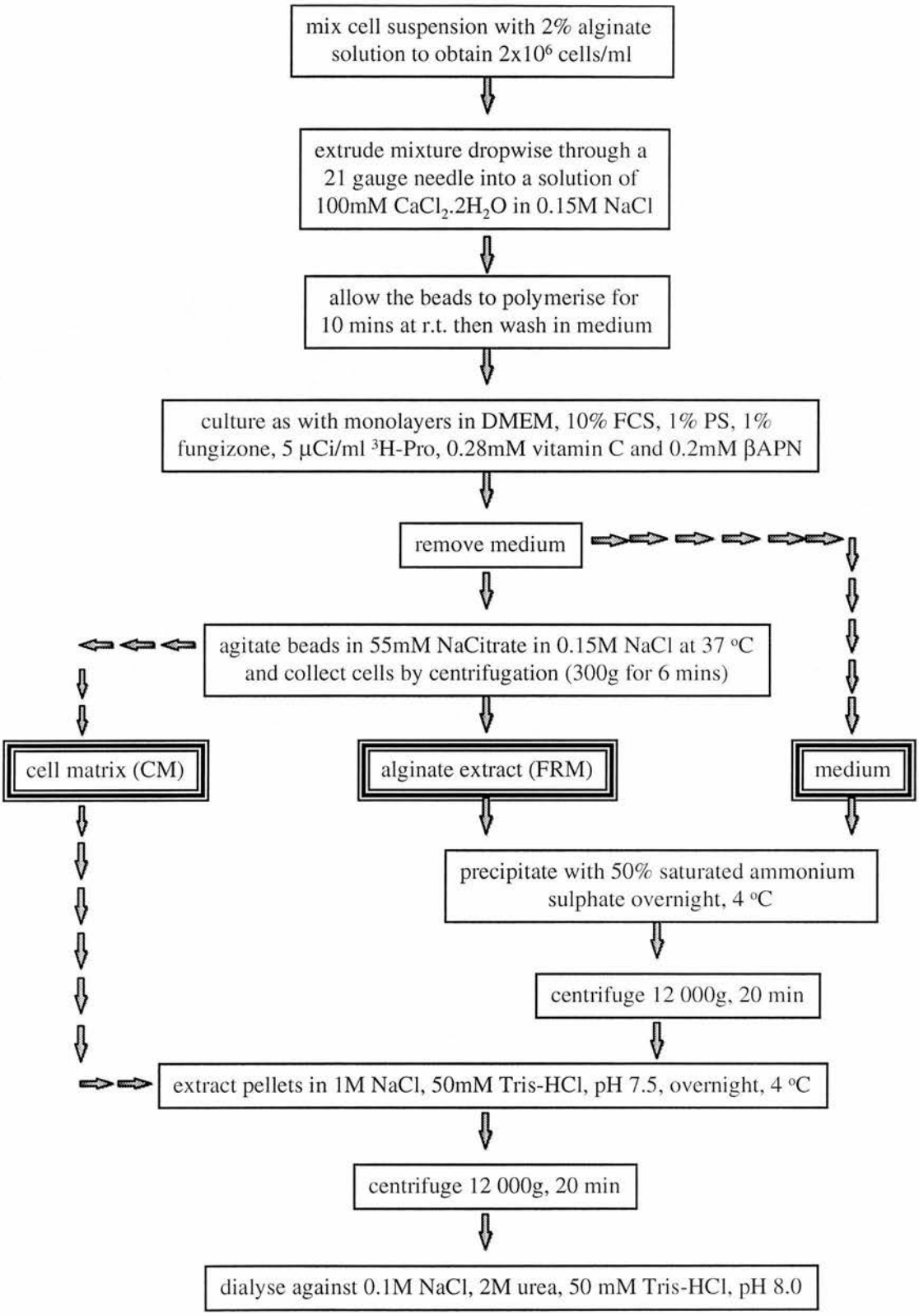


Figure 2.1. Flow diagram summarising the partial purification of collagens from alginate bead culture. Refer to text for further details (sections 2.2.2 and 2.2.4).

2.2.4 Partial Purification of Cartilage Collagens

The partial purification of cartilage collagens is summarised in Figure 2.1. Proteins in the medium and FRM were precipitated by the addition of ammonium sulphate (50% saturation) in the presence of the proteinase inhibitors, followed by shaking at 4 °C overnight on a rotary shaker. The samples were centrifuged at 12 000g for 20 mins and the pellet re-suspended in extraction buffer (1M NaCl, 50mM Tris-HCl, pH 7.5) in the presence of proteinase inhibitors. All samples, including the CM, were extracted by incubation with constant agitation at 4 °C overnight. After centrifugation for 20 mins at 12 000g to remove any insoluble material, the samples were dialysed extensively against 0.1M NaCl, 50mM Tris-HCl, 2M urea, pH 8.0 with proteinase inhibitors to remove unincorporated radioisotope.

2.2.5 Pepsin Digestion of Collagens

Samples were dialysed against 0.5M acetic acid and digested with pepsin (100 μg/ml) overnight at 4 °C with constant agitation. After neutralising each sample with 5M sodium hydroxide (NaOH), the collagens were precipitated by standing overnight at 4 °C in the presence of 10% (v/v) trichloroacetic acid (TCA). Collagens were pelleted by centrifugation at 11 000g for 30 mins then resuspended in SDS-PAGE sample buffer (0.125M Tris-HCl, 2% (w/v) SDS, pH 6.8).

The collagens were separated by SDS-PAGE with 6% separating and 4.5% stacking gels (both reducing and non-reducing conditions), loading an equivalent volume in each lane. The gels were examined by fluorography and the relative molar percentage of each collagen chain was measured by densitometry (Chromoscan 3, Joyce Loebl), correcting for the differing molecular weights of each collagen type as follows: collagen XI values (total α1(XI) + α2(XI) bands) were increased by one third due to the co-migration of the α3(XI) and the α1(II) chains, with the corresponding amount deducted from the α1(II) band; collagen IX values (total COL2/COL3 and COL3 bands) were increased by one third as after pepsin digestion the molecule is two

thirds its original molecular weight (Bruckner *et al.*, 1985) and by another third as the molecule is two thirds the length of collagens II or XI (Vaughan *et al.*, 1988).

The distribution of collagens in each compartment was confirmed by western blotting with affinity purified polyclonal antibodies to collagens II and IX. Full details of the western blotting technique can be found in section A.3. The dilutions of both the primary and secondary antibody (horse-radish peroxidase conjugated goat anti-rabbit) was 1:3000. Blots were detected by enhanced chemiluminescence (ECL; section A.3.2).

To determine the ratios of collagens II, IX and XI in the combined CM and FRM, the average total collagen produced each day (Figure 2.5) and the ratios of the collagens produced in each of these compartments (Table 2.1) were utilised. For example, if the average total collagen in the CM was TC μg, that of the FRM was TF μg, and the medium TM μg and the collagen II/IX/XI ratio was a/b/c in the CM, a`/b`/c` in the FRM and a``\b``\c`` n the medium, the calculation to work out the ratio of collagen II in (1) the combined CM/FRM or (2) the CM/FRM/medium was as follows:

$$1.\quad \frac{(a \times TC) + (a` \times TF)}{TC + TF} \qquad 2.\quad \frac{(a \times TC) + (a` \times TF) + (a`` \times TM)}{TC + TF + TM}$$

This equation was used to determine the ratios of each collagen type in both the presence and the absence of βAPN.

2.2.6 Analysis of Collagen IX From The Medium

The presence of the non-proteoglycan and proteoglycan forms of collagen IX were assessed according to Yada *et al.* (1990, 1992). Samples of partially purified collagens from the medium were dialysed against start buffer (20mM NaCl, 2M urea, 50mM Tris-HCl, 1mM EDTA, pH 8.0). After the addition of 0.5% (w/v) CHAPS, samples were loaded onto a DEAE-Sephacel column (1cm x 6cm) which had been pre-equilibrated with start buffer containing 0.5% (w/v) CHAPS (equilibration buffer) and 1ml fractions were collected. The column was then washed with 20ml of

equilibration buffer, followed by elution of bound proteins with a linear gradient (60ml) from 20mM to 0.6M NaCl in equilibration buffer. Finally, the column was washed with 10ml of 2M NaCl in equilibration buffer. Every fourth fraction (10µl) was added to 3ml of Ultima Gold liquid scintillation cocktail (Packard) and ^{3}H cpm were determined using a Packard 1900 CA liquid scintillation analyser.

Fractions were pooled as indicated (Figure 2.6) and precipitated by addition of 3 volumes of 95% (v/v) ethanol containing 1% (w/v) potassium acetate. The pooled fractions were subject to SDS-PAGE and fluorography (5% separating and 4% stacking gels, non-reducing conditions) with or without prior digestion with chondroitinase ABC (0.1 unit.ml^{-1}) for 2 hours at 37 °C. Molecular weights were determined from Coomassie stained gels containing high molecular weight markers (cross-linked phosphorylase b).

2.2.7 Quantitation of Sulphated GAG's

Alginate beads and culture medium were assessed for the presence of sulphated GAG's according to the method of Enobakhare *et al.* (1996). On days 1, 4, 7 and 14, alginate beads (approx. 1ml) and media (5ml) were collected from cultures grown in the presence or absence of 0.2mM βAPN. Four volumes of 55mM sodium citrate in 0.15M NaCl, 5mM cysteine hydrochloride, 5mM EDTA containing 0.2 mg/ml papain was added to the alginate beads for 16 hours at 60 °C. Samples (40µl) of the papain digests and the media were then added to the wells of a 96 well plate (Dynatech). To each experimental well, and to wells containing known concentrations of chondroitin sulphate, 250µl of 1,9-dimethylmethylene blue dye (DMB) in 0.02% ethanol, 29.4mM sodium formate, pH 1.5 was added. The absorbance of each well was measured immediately at 490 nm on a plate reader (Dynatech) and the concentration of a control sample containing alginate alone was subtracted from all experimental values.

2.2.8 Quantification of Total Collagen

Total collagen content was measured by determination of hydroxyproline content in each sample (Woessner, 1961; modified by Robins *et al.*, Rowett Research Institute, Aberdeen, personal communication). Briefly, aliquots of samples were hydrolysed in 6M HCl for 16 hours at 110 °C and the hydrolysates desiccated to dryness. Following the addition of 1ml H_2O, 100µl of each of the rehydrated samples were incubated for 5 mins at room temperature in the presence of 2% (w/v) chloramine T and concentrated 2-methoxyethanol. Following the addition of 12.5% (w/v) dimethylaminobenzaldehyde in 12M HCl, the samples were incubated for 20 mins at 60 °C to allow the colorimetric reaction to proceed. The absorbance of each sample was measured using a spectrophotometer (553 nm) and a standard curve was generated using known concentrations of hydroxyproline (0, 1.25, 2.5, 5, 10 and 15 µM). The total amount of collagen was calculated assuming a hydroxyproline content of 10% (w/w; Berg, 1982). N.B. This has since been shown to be an underestimate (should assume 14%) as collagen IX has a hydroxyproline content of 19%.

2.2.9 Analysis of Collagen XI in the CM

Samples of the supernatant of a 1M NaCl extract of the CM (day 14) in the absence of βAPN were digested with pepsin as described previously (section 2.2.5). After neutralising with 1M NaOH, proteins were precipitated by addition of 3 volumes of 95% (v/v) ethanol, 1% (w/v) potassium acetate. After centrifugation (5600g for 10 min), the pellets were resuspended in storage buffer (0.4M NaCl, 0.1M Tris-HCl, pH 7.4). The insoluble pellet that remained after 1M NaCl extraction was also digested with pepsin, precipitated with ethanol and resuspended in storage buffer as above. Not all proteins, however were soluble in the storage buffer so further centrifugation gave rise to pepsin-soluble material (supernatant) and pepsin-insoluble material (pellet). The pepsin-insoluble sample was further extracted for 3 mins at 100 °C in 2% (w/v) SDS, 0.125M Tris-HCl, pH 6.8. Equivalent proportions of each of three samples were thus analysed by SDS-PAGE: the soluble proteins of the 1M NaCl extract, the pepsin-soluble proteins of the original insoluble pellet and the SDS

extract of pepsin-insoluble proteins. SDS-PAGE gels (6% separating and 4.5% stacking gels, under non-reduced conditions) were examined by fluorography and exposed to Kodak XAR film at -70 °C before densitometry revealed the ratios of collagens in each sample. Corrections were made for the differing molecular weight of each collagen type as described previously (section 2.2.5).

2.3 RESULTS

2.3.1 Biochemical Analysis of Alginate Bead Culture

An indicator of phenotypic stability of chondrocytes in culture is the production of collagen II and other cartilage collagens (IX, X and XI). Therefore, the first assay investigates the collagen types produced by chondrocytes in alginate bead culture and their distribution in each compartment of the culture system. Collagen IX is subject to further analysis to determine whether it is present as a non-proteoglycan and/or a proteoglycan form and the production and distribution of total proteoglycan is also examined to provide further information on the composition of the ECM.

2.3.1.1 Analysis of Crude Extract

Chondrocytes cultured in alginate beads in the absence of βAPN were metabolically labelled with L-[5-^{3}H]proline as described previously (section 2.2.2). Following digestion with pepsin (section 2.2.5), collagens present in the CM were analysed by fluorography and western blotting with polyclonal antibodies to collagens II, IX and X (Figure 2.2). It was found that collagens II, X and XI were present in the CM but collagen IX was absent. This was surprising, as collagen IX in cartilage is covalently cross-linked to collagen II fibrils, so a more in depth analysis of the collagens present in each compartment of the culture system was carried out.

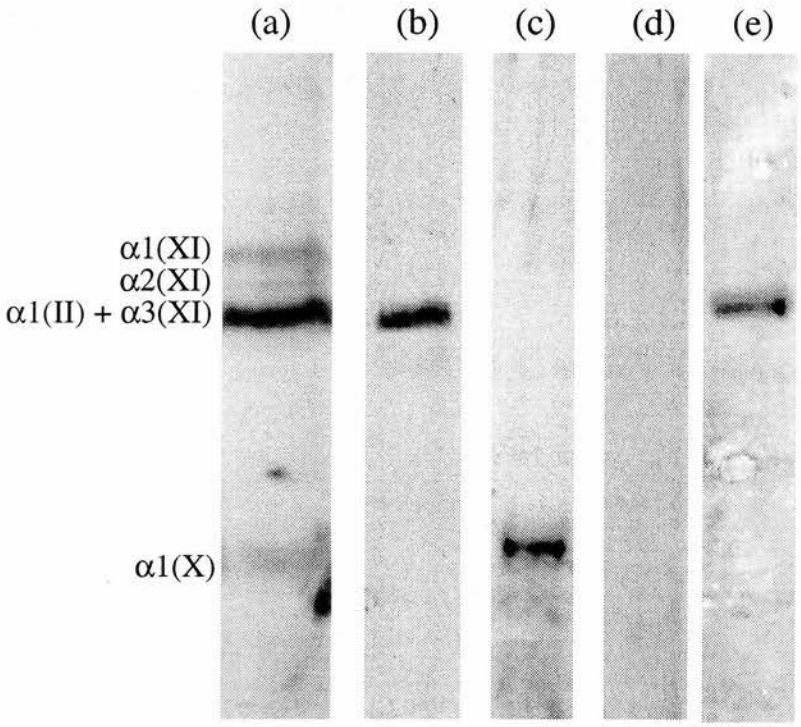


Figure 2.2. Analysis of collagens present in a crude extract of the cell-associated matrix (CM) of alginate bead culture. (a) Fluorogram showing the presence of collagens II, X and XI on day 7 of the culture period while collagen IX was absent. Western blots with polyclonal antibodies (1:3000 dilution) confirmed the presence of collagens II (b) and X (c) and the absence of collagen IX (d) in the CM. (e) Coomassie stained, pepsin digested collagen II standard.

2.3.1.2 Distribution of Collagens in Each Compartment

Analysis of pepsinised collagens by fluorography showed that collagen II was found in all three compartments of the culture system, i.e. the cell-associated matrix (CM), the further-removed matrix (FRM) and the medium (Figure 2.3a-c). Collagen IX was recovered from the FRM and in large amounts from the medium, but not detected in the CM. The distribution of the collagen types in each compartment was confirmed by western blotting with affinity purified polyclonal antibodies to either collagen II or collagen IX (Figure 2.4). The affinity purified collagen II antibodies showed several bands in the CM and FRM in both the presence and the absence of βAPN. This is probably due to breakdown products rather than cross-reactivity of the antibodies with collagen IX, as there are more than two bands present and there is no cross-reactivity with collagen IX in the medium (Figure 2.4).

Analysis of hydroxyproline showed that total collagen in the CM remained relatively stable over the 7 day culture period (315 μg collagen on day 1 to 285 μg on day 7; Figure 2.5a) with the FRM showing only a slight increase (135 μg on day 1 to 185 μg on day 7). In the accumulated medium however, there was a large increase in total collagen (347 μg on day 1 to 2332 μg on day 7), when taking account of daily collections of medium. Similar results were obtained when analysing total non-dialysable cpm over time (results not shown).

Densitometry of each fluorogram revealed the ratio of collagens II:IX:XI in each compartment of the culture system (Table 2.1). Collagen II comprised 97% of total collagen in the CM, 75% in the FRM and 40% in the medium. Collagen XI made up 3% of the CM and 11% of the FRM while collagen IX comprised 14% of the collagens present in the FRM and 60% in the medium. Expressing the ratios of II, IX and XI as a percentage of total collagen (CM + FRM) produced each day revealed that the matrix surrounding the chondrocytes in alginate bead culture comprised 91% collagen II, 4% collagen IX and 5% collagen XI. This is similar to the 8:1:1 ratio of collagens II:IX:XI found *in vivo* (Vaughan *et al.*, 1988). The ratio (II/IX/XI) of total collagen produced in all three compartments was 58/48/1. The high amount of

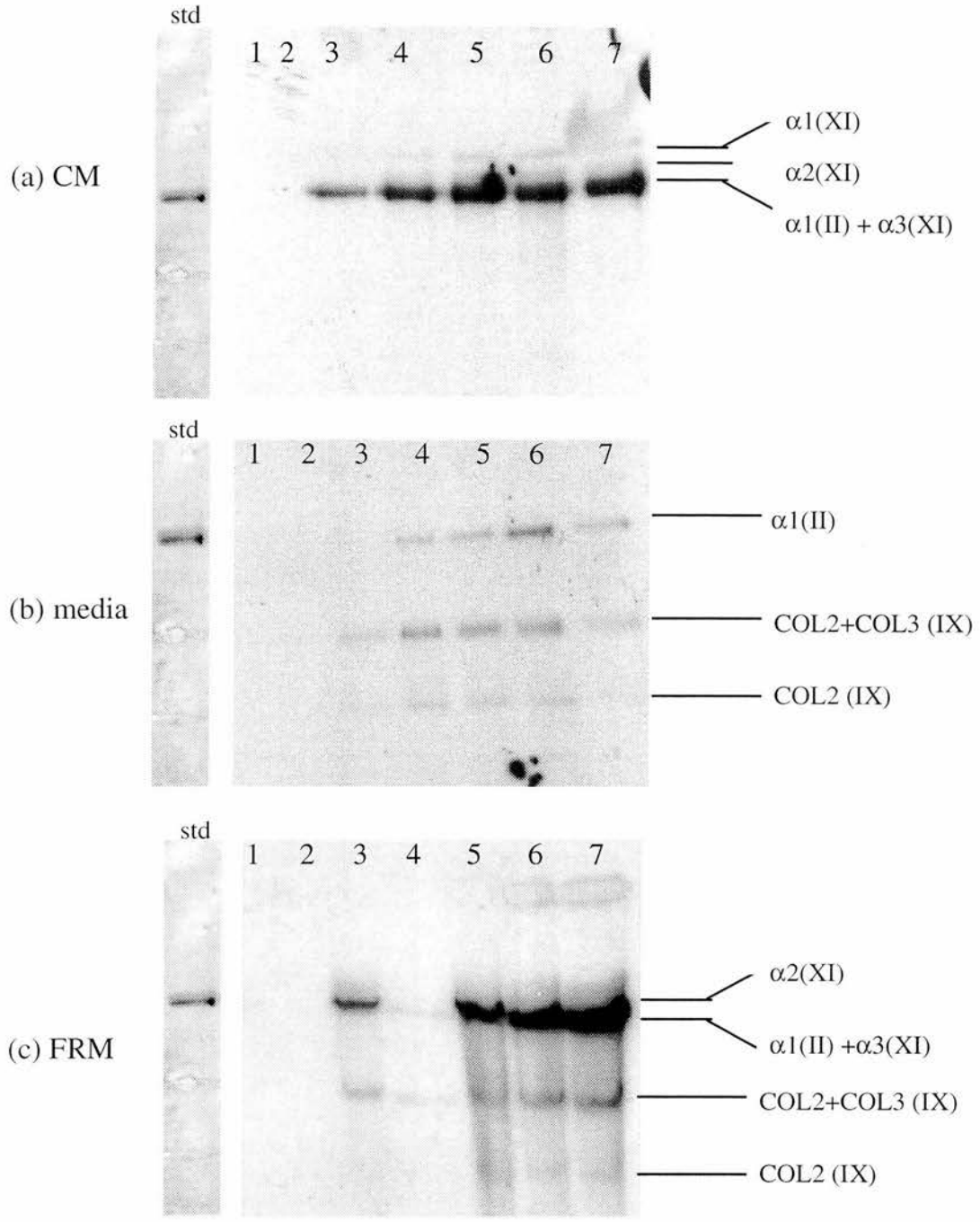


Figure 2.3. Distribution of collagens in alginate culture, after pepsin treatment, in the absence of βAPN. Fluorograms of SDS-PAGE (6% reduced gels) show that collagen II is found in all three compartments i.e. the cell associated matrix (CM; (a)), the medium (b) and the further-removed matrix (FRM; (c)). Collagen XI is found in the CM and the FRM while collagen IX is present in the FRM and the medium. Std represents Coomassie stained, pepsinised collagen II standard while numbers indicate days in culture.

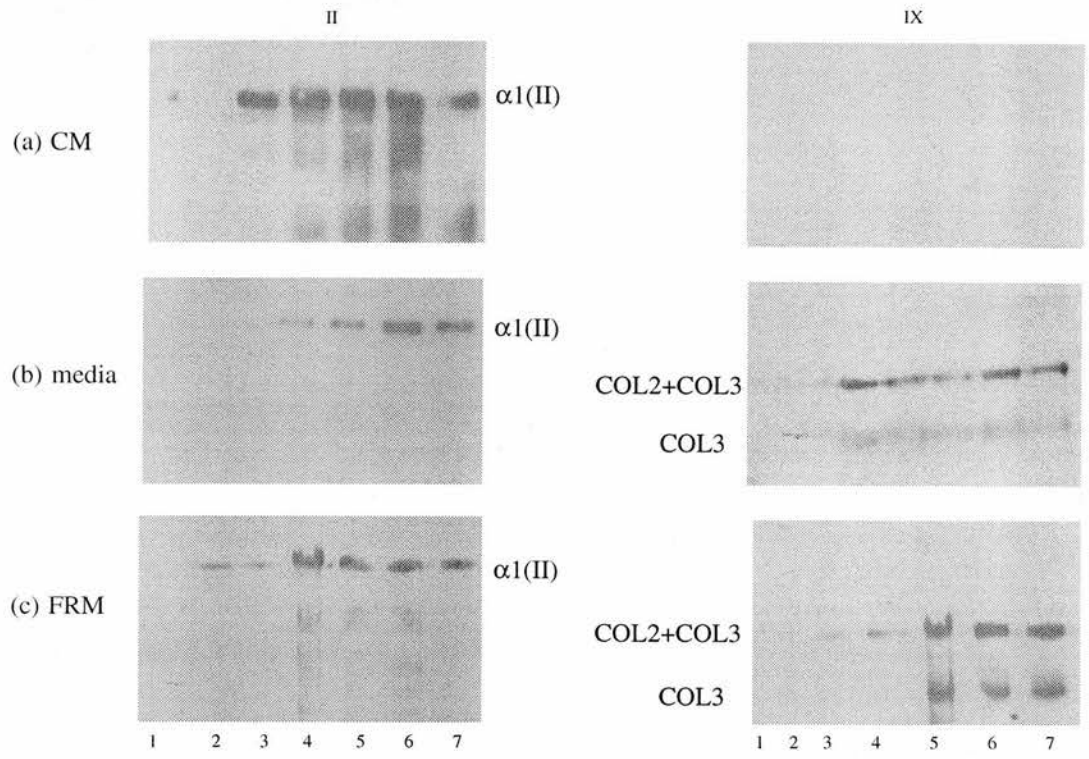


Figure 2.4. Western blots with antibodies to either collagen II or IX confirms the distribution of collagens in each compartment in the absence of βAPN. Samples were digested with pepsin before SDS-PAGE and then blotted onto nitrocellulose. Polyclonal antibodies were affinity purified and used at a dilution of 1:3000. The secondary antibody was used at a dilution of 1:3000 and blots were detected by ECL. Blots were stripped and reprobed using the other antibody and the film was cut to the same size so direct comparisons could be made.

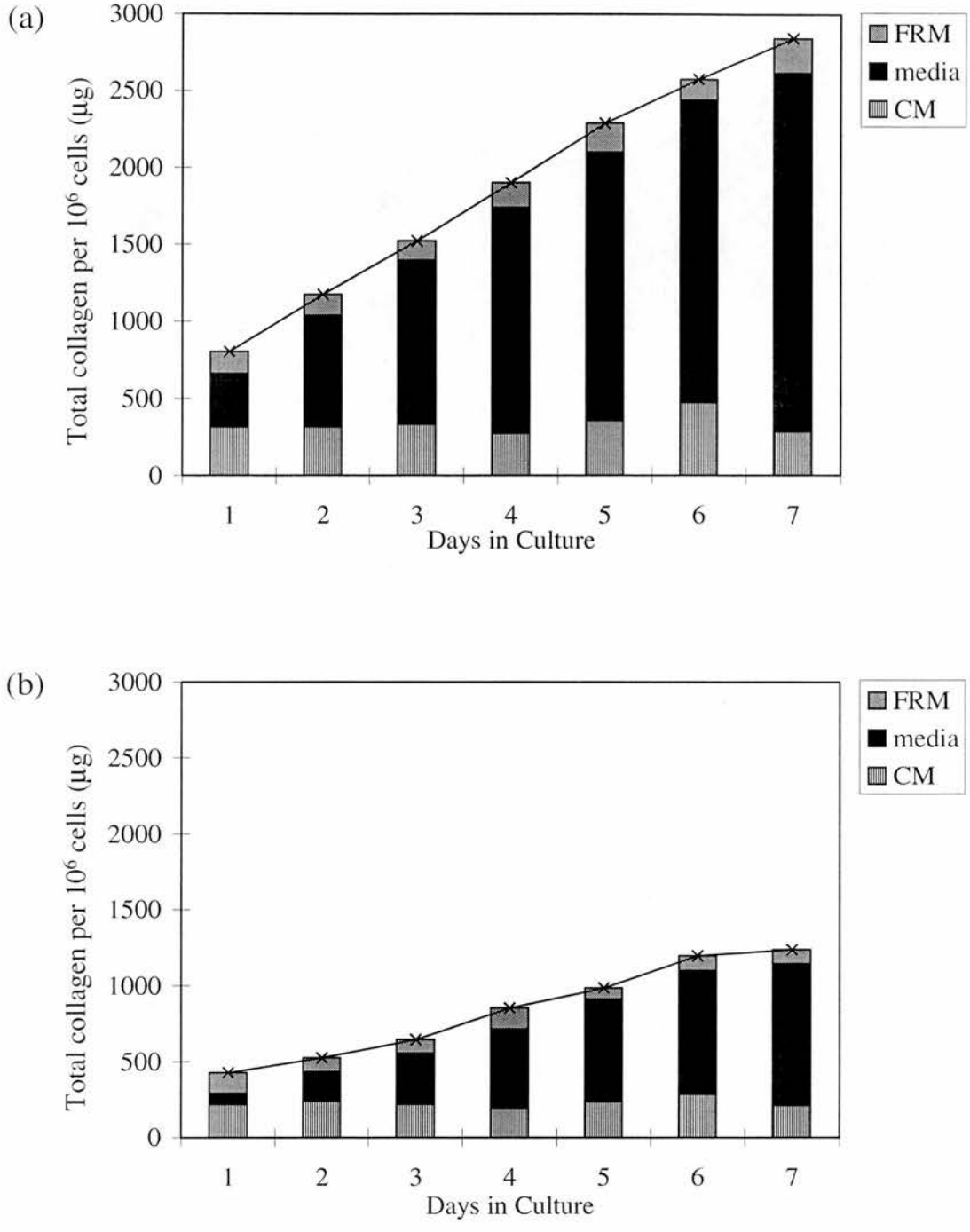


Figure 2.5. Total collagen produced in (a) the absence and (b) the presence of βAPN. (a) Collagen production in the CM and FRM remains relatively constant over the 7 day culture period whereas there is a large increase in the amount recovered from the medium. (b) βAPN reduces the collagen present by approx. 50% in each compartment. Media fractions represent cumulative amounts, taking into account daily collections of media.

	-βAPN			+βAPN		
	II	**IX**	**XI**	**II**	**IX**	**XI**
CM	97	-	3	93	-	7
FRM	75	14	11	77	10	13
MEDIA	40	60	-	40	60	-
combined CM/FRM	91	4	5	88	2	10
combined CM/FRM/media	51	48	1	64	31	5

Table 2.1 Ratio of collagen types present in each compartment in the presence and absence of βAPN. Data represents an average over 7 days. Corrections were made for the differing molecular weights of each collagen type as described in section 2.2.5.

collagen IX produced compared to cartilage was surprising but may be accounted for if there are losses of collagen IX *in vivo* due to diffusion out of the tissue.

2.3.1.3 Analysis of Collagen IX from the Medium

Chicken, bovine and human chondrocytes cultured *in vitro* synthesise collagen IX in both non-proteoglycan and proteoglycan forms due to the attachment of a single chondroitin sulphate glycosaminoglycan (GAG) side-chain to α2(IX) (Huber *et al.*, 1986; Bruckner *et al.*, 1988; Ayad *et al.*, 1991; Yada *et al.*, 1990). To test which form of collagen is produced by chick chondrocytes in alginate bead culture, medium was analysed by ion exchange chromatography and chondroitinase ABC digestion. DEAE-Sephacel column chromatography of collagen IX recovered from the medium showed that, following a gradient from 20mM to 0.6M NaCl, ^{3}H radioactivity was largely separated into 3 peaks (Figure 2.6). Fractions were pooled as indicated (A, B, C and D), and some samples were digested with chondroitinase ABC which removes dermatan sulphate and chondroitin sulphate GAG chains (Yada *et al.*, 1990). The unbound proteins (fraction A) showed a major sharp band at 270 kDa (Figure 2.6b) which did not change following digestion with chondroitinase ABC. Fraction B contained a band which may represent the pN-precursor form of collagen II (see chapter 4), but no bands corresponding to collagen IX. Fraction C showed a diffuse band (~300 kDa; Figure 2.6b) that, following digestion with chondroitinase ABC increased mobility on SDS-PAGE to 270 kDa (Figure 2.6c). The removal of the GAG chain from α2(IX) resulted in its co-migration with the α3(IX) chain, which is shown by the increased intensity on the fluorogram (Figure 2.6d). The proteoglycan form of collagen IX represents 80% of the total collagen IX produced while the non-proteoglycan form represents 20%. These results indicate that both a non-proteoglycan (270 kDa) and a proteoglycan (~300 kDa) form of collagen IX are produced by chick chondrocytes cultured in alginate beads which is similar to collagen IX purified from cartilage (Yada *et al.*, 1992).

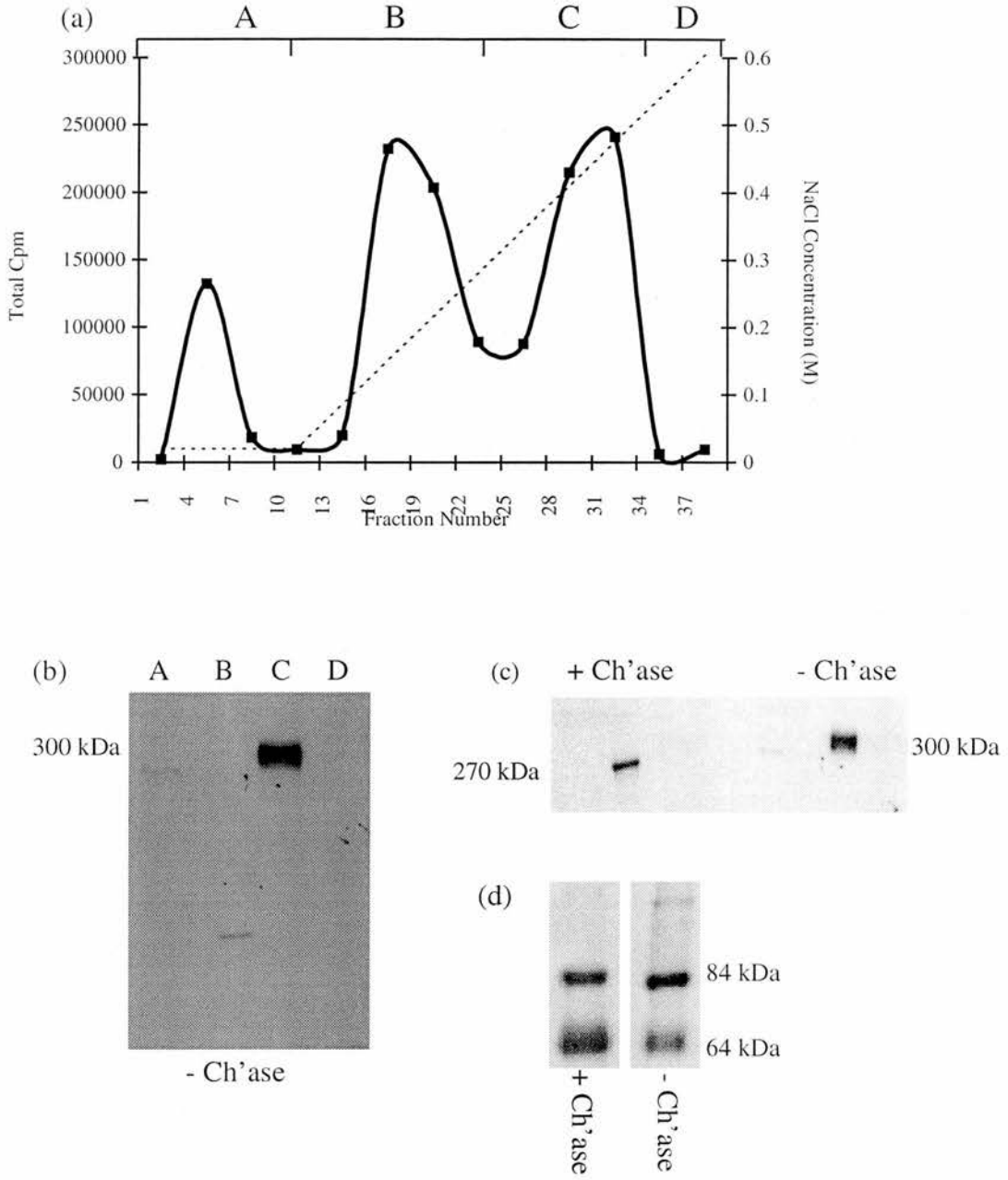


Figure 2.6. Analysis of collagen IX secreted into the medium. (a) elution profile of a DEAE-Sephacel column (gradient: 20mM to 0.6M NaCl). (b) 5% SDS-PAGE gel (non-reduced conditions) shows the presence of 2 forms of collagen IX (fractions A and C). (c) 5% SDS-PAGE gel (non-reduced conditions) of a sample of fraction C with or without prior digestion with chondroitinase ABC shows that digestion with chondroitinase ABC results in an increased mobility on SDS-PAGE. (d) 5% SDS-PAGE gel (reduced conditions) of fraction C with or without prior digestion with chondroitinase ABC shows that the α2(IX) chain co-migrates with the α3(IX) chain (64 kDa).

2.3.1.4 Quantification of Sulphated GAG's

To determine the concentration of proteoglycan produced by chick chondrocytes in alginate beads, the sulphated GAG concentration was measured using the method of Enobakhare *et al.* (1996). The amount of proteoglycan remained constant in the combined CM/FRM over the 14 day culture period whereas there was a constant increase in the amount of proteoglycan in the medium (Figure 2.7). The stable concentration of proteoglycan recovered from the CM/FRM is indicative of proteoglycan aggregation, while the increase in the medium probably represents subunits or fragments of proteoglycans that have been turned-over. Total proteoglycan (CM/FRM + medium) increased from approx. 3.5 μg SGAG/10^6 cells on day 1 to 9 μg SGAG/10^6 cells on day 14 (Figure 2.13), although it should be noted that this figure represents quadruplicates of one experiment and thus the increase in the amount of proteoglycan may not be indicative of further experiments.

2.3.2 Effect of βAPN on ECM Production

β-aminopropionitrile (βAPN) is a potent irreversible inhibitor of lysyl oxidase, the enzyme that initiates cross-link formation in elastin and collagen (Tang *et al.*, 1983). The inhibitor is purified from the sweet pea *Lathyrus odoratus*, ingestion of which causes the condition lathyrism. βAPN has been used to increase solubility and thus allow easier extraction of collagen from both tissues and cell culture systems (Levene and Gross, 1959). Traditionally, βAPN has been added to the medium of alginate bead cultures although nothing is known of its effect on the chondrocytes or the ECM produced so the effect of this cross-link inhibitor was investigated. A summary of the following results can be found in Table 2.2.

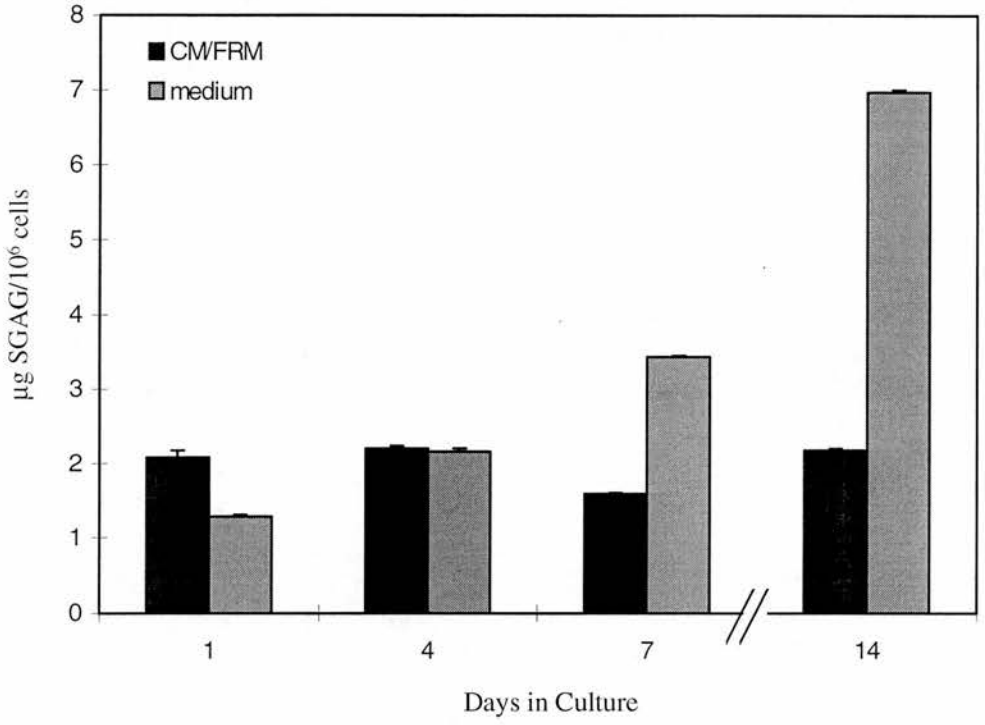


Figure 2.7. Proteoglycan produced by chick chondrocytes in alginate bead culture in the absence of βAPN. On the days indicated alginate beads (CM + FRM) were digested with papain and medium was collected. The content of proteoglycan in each compartment was quantified by the 1,9-dimethylmethylene blue assay as described in section 2.2.7. Medium values represent accumulated medium. Error bars = ± standard error, n=4 replicates from 1 experiment.

2.3.2.1 Total Cell Numbers

The presence of βAPN in the culture medium had little effect on cell numbers (Figure 2.8). In both culture conditions, chondrocytes show mild proliferation, increasing from approx. $2x10^7$ cells on day 1 to $4x10^7$ cells on day 14. Although viability was not assessed by Trypan blue and thus firm conclusions cannot be drawn, the increase in cell numbers suggests that βAPN is not toxic to chondrocytes.

2.3.2.2 Effect of βAPN on Collagen Synthesis

Analysis of pepsinised collagens by fluorography showed that in the presence of βAPN collagen II was found in all three compartments of the culture system (Figure 2.9a-c). Collagen IX was found in the FRM and in large amounts in the medium but was absent from the CM. Collagen XI was detected in the CM and the FRM but was not found in the media. The distribution of collagen types in each compartment was confirmed by western blotting with affinity purified polyclonal antibodies to either collagen II or collagen IX (Figure 2.10a-c). These results are similar to those in the absence of βAPN but the ratios of II:IX:XI differed in each condition (Table 2.1).

Hydroxyproline analysis showed that the amount of collagen present in each compartment was reduced by approx. 50% when βAPN was added to the culture medium (Figure 2.5b). A similar effect of βAPN was seen in total, non-dialysable cpm (results not shown). Although there was a decrease in the amount of collagen recovered, βAPN did not affect the ratio of collagens II:IX present in the medium (40:60; Table 2.1) but the production of collagen XI appeared to be greater in the presence of βAPN (7%) than in its absence (3%; Table 2.1; section 2.3.2.3). Expressing the ratios of II, IX and XI as a percentage of total collagen (CM + FRM) produced each day revealed that the matrix produced in the presence of βAPN comprised 88% collagen II, 2% collagen IX and 10% collagen XI. Full details of the calculation can be found in section 2.2.5.

In summary, βAPN reduced total collagen production by 50% but the distribution of the collagen types in each compartment did not alter. There was, however an

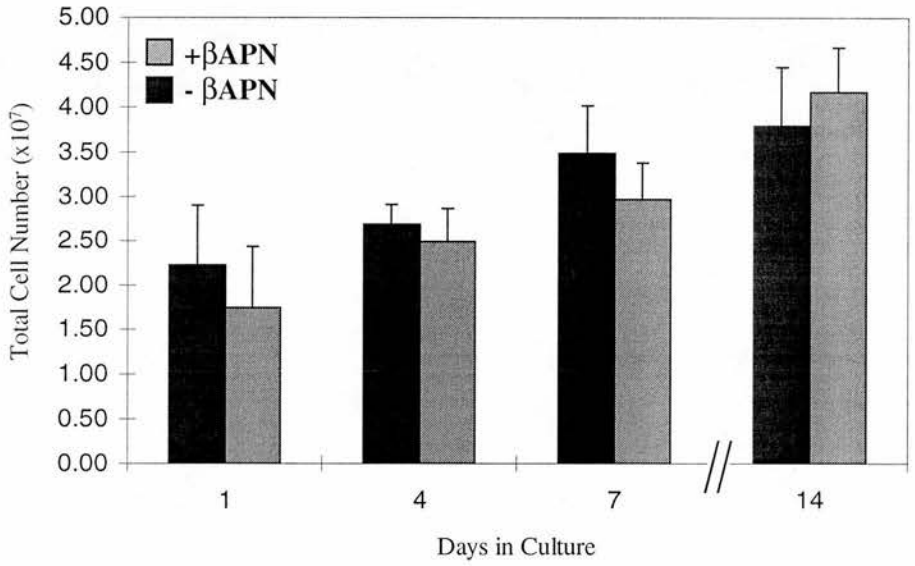


Figure 2.8. Total cell numbers in the presence and absence of βAPN. Cell numbers were calculated using a haemocytometer. Error bars = ± standard error, n=4 (independent experiments).

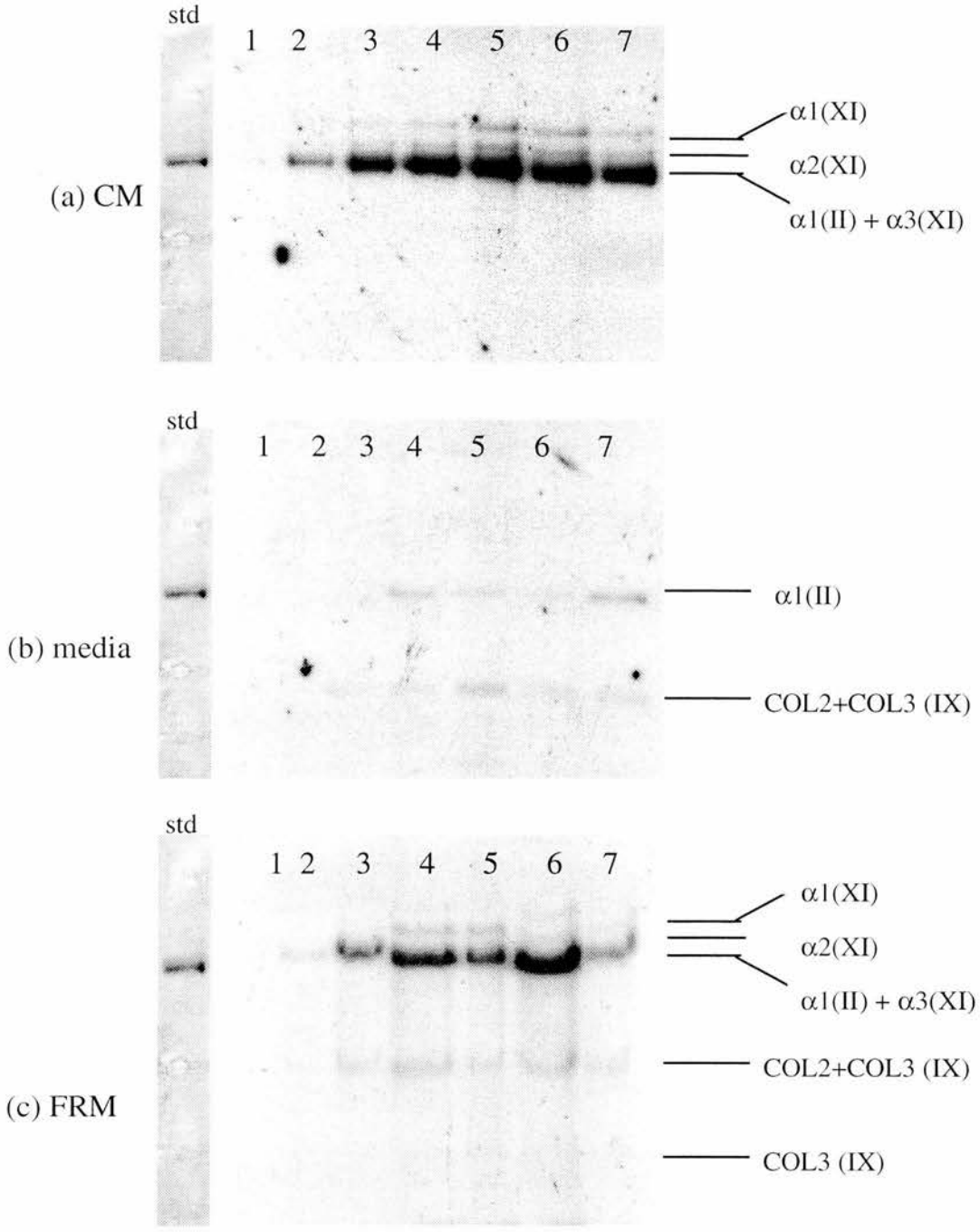


Figure 2.9. Distribution of pepsinised collagens in alginate culture in the presence of βAPN. Fluorograms of SDS-PAGE (6% reduced gels) show that collagen II is found in all three compartments i.e. the CM (a), the medium (b) and the FRM (c). Collagen XI is found in the CM and the FRM while collagen IX is present in the FRM and the medium. Std represents Coomassie stained pepsinised collagen II standard while numbers indicate days in culture.

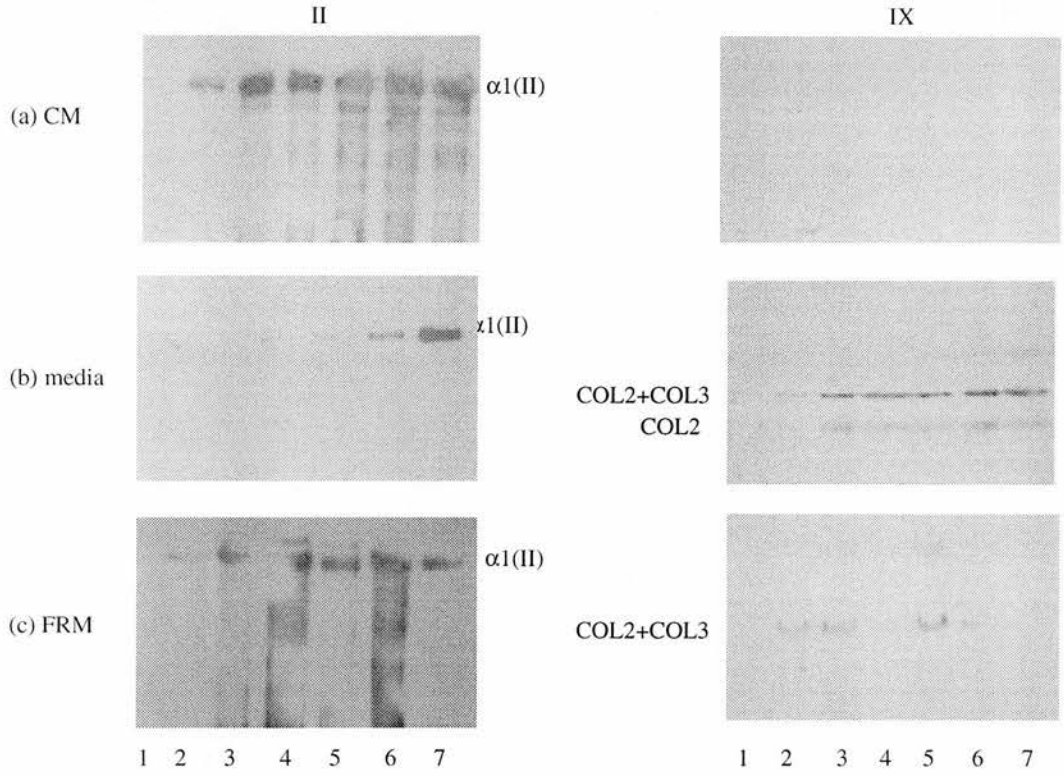


Figure 2.10. Western blots with antibodies to either collagen II or IX confirms the distribution of these collagens in each compartment in the presence of βAPN. Samples were digested with pepsin before SDS-PAGE and then blotted onto nitrocellulose. Polyclonal antibodies were affinity purified and used at a dilution of 1:3000. The secondary antibody was used at a dilution of 1:3000 and blots were detected by ECL. Blots were stripped and reprobed using the other antibody and the film was cut to the same size so direct comparisons could be made.

apparent increase in the amount of collagen XI recovered from the CM in the presence of βAPN.

2.3.2.3 Analysis of Collagen XI in the CM

Relatively higher amounts of collagen XI were present in the CM in the presence of βAPN (7%) than in the absence of βAPN (3%; Table 2.1). To examine whether this was due to the relative insolubility of collagen XI as a result of cross-linking, the insoluble pellet that remained after extraction of the CM was analysed for the presence of collagens by pepsin digestion and SDS extraction.

On day 14, in the absence of βAPN, collagen II was present in the supernatant of the 1M NaCl extract in small quantities (Figure 2.11a). Pepsin digestion of the insoluble pellet which remained after neutral salt extraction revealed the presence of more collagen II while a SDS extract of the pepsin insoluble material showed the presence of collagens II, IX, X and XI. These distributions were confirmed by western blotting with affinity purified polyclonal antibodies against collagens II, IX and X (results not shown). Densitometry revealed the ratios of the different collagens in both the 1M NaCl supernatant and the insoluble pellet and showed that in the absence of βAPN, collagen II comprised 72% of collagen present in the combined three lanes while collagens IX, X and XI represented 20%, 1% and 7% respectively. By contrast to the above results, it was found that all collagens produced in the presence of βAPN were soluble in the 1M NaCl extraction buffer (Figure 2.11b). Collagens IX and X were not detected in the presence of βAPN.

2.3.2.4 Quantitation of Sulphated GAG's in the Presence of βAPN

In the presence of βAPN, proteoglycans in the CM/FRM showed a steady decrease over the culture period, while there was a concomitant increase in the medium (Figure 2.12). This indicates that proteoglycan aggregation in the CM/FRM was impaired. Total proteoglycan (CM/FRM + medium) increased from approx. 5 μg

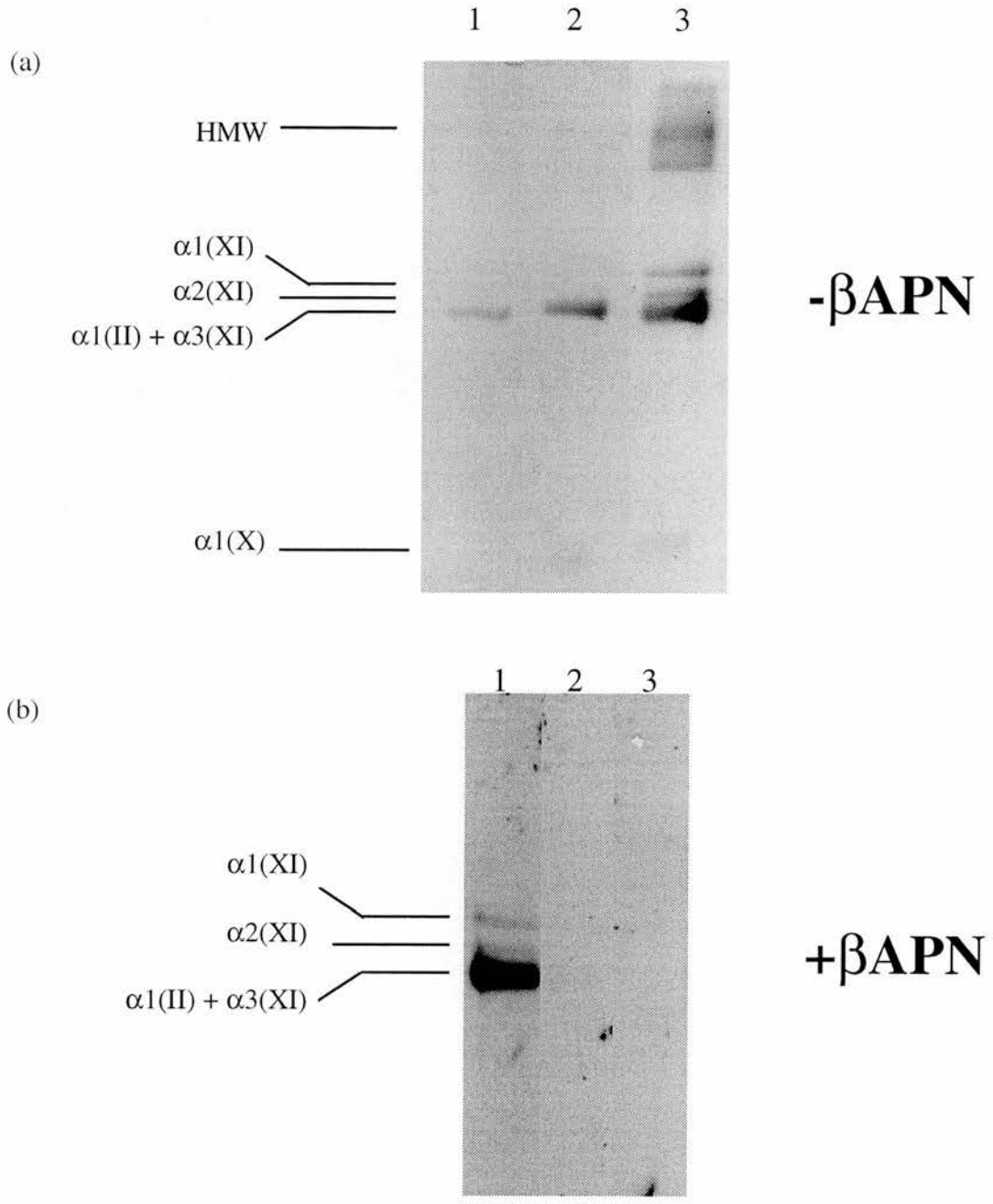


Figure 2.11. Analysis of collagen XI in the CM. On day 14 the CM was resuspended in 1M NaCl extraction buffer. *Lane 1* pepsin digestion of the 1M NaCl supernatant. *Lane 2* pepsin digestion of the 1M NaCl insoluble pellet. *Lane 3* SDS extract of pepsin insoluble material (non-reduced). (a) In the absence of βAPN a small amount of collagen II is recovered from the 1M NaCl supernatant while further collagen II and collagens IX, X and XI are recovered in the SDS extract of pepsin insoluble material. (b) The presence of βAPN allows recovery of collagen XI in the 1M NaCl supernatant.

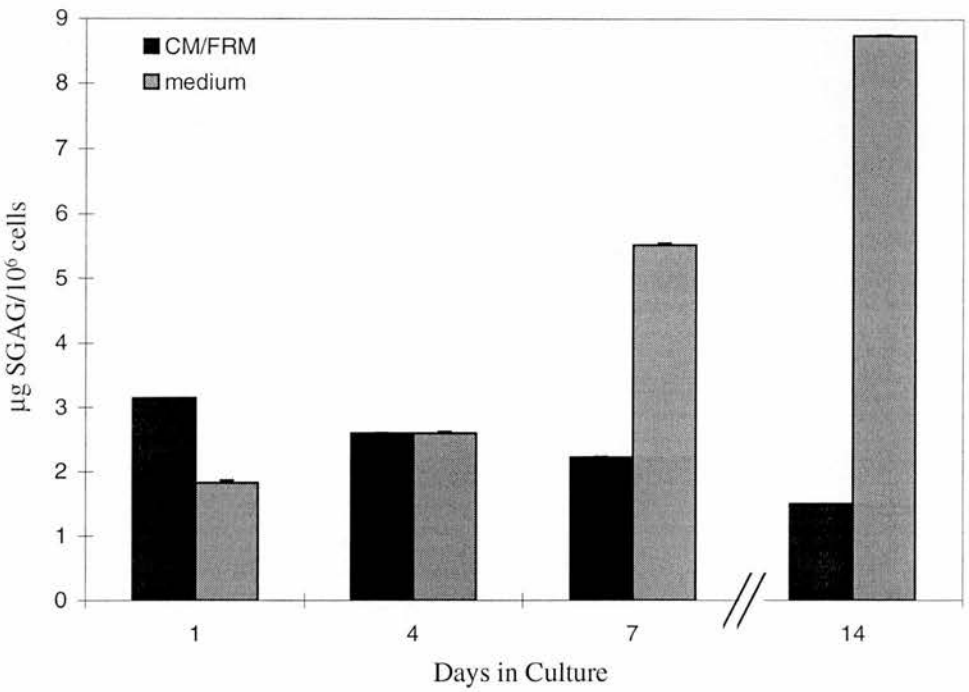


Figure 2.12. Proteoglycan produced by chondrocytes in alginate beads in the presence of βAPN. On the days indicated alginate beads (CM + FRM) were digested with papain and medium was collected. The content of proteoglycan in each compartment was quantified by the 1,9-dimethylmethylene blue assay as described in section 2.2.7. Medium values represent accumulated medium. Error bars = ± standard error, n=4 replicates of 1 experiment.

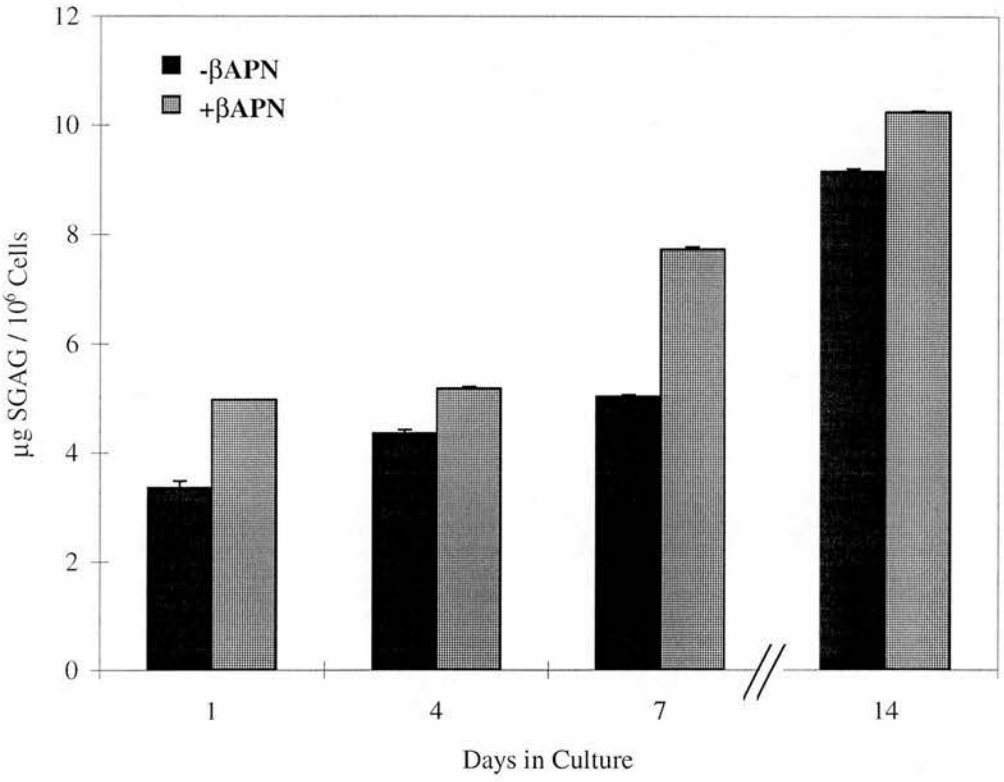


Figure 2.13. Total proteoglycan produced by chick chondrocytes in alginate bead culture in the presence and absence of βAPN. On the days indicated, the total proteoglycan (CM/FRM + medium) produced by chondrocytes in alginate beads was quantified by the 1,9-dimethylmethylene blue assay as described in section 2.2.7. Medium values represent accumulated medium. Error bars = ± standard error, n=4 replicates of 1 experiment.

	- βAPN	+ βAPN
Total Cell Numbers	$3.75x10^7$ on day 14	$4.1x10^7$ on day 14
Distribution of Collagen Types	collagen II in CM, FRM and medium collagen IX in FRM and medium collagen XI in CM (small amounts) and FRM	collagen II in CM, FRM and medium collagen IX in FRM and medium collagen XI in CM and FRM
Ratios (II/IX/XI)	CM - 97/0/3 FRM - 75/14/11 medium - 40/60/0	CM - 93/0/7 FRM - 77/10/13 medium - 40/60/0
Ratio (II/IX/X/XI) in SDS Extracted Pellet	72/20/1/7	0/0/0/0
Total Collagen	$2.8x10^3$ μg/10^6 cells on day 7	$1.2x10^3$ μg/10^6 cells on day 7
Total Proteoglycan	5μg SGAG/10^6 cells on day 7	8μg SGAG/10^6 cells on day 7

Table 2.2. Summary of βAPN results.

SGAG/10^6 cells on day 1 to 10 μg SGAG/10^6 cells on day 14 (Figure 2.13). Consistently higher amounts of total proteoglycan (CM/FRM + medium) were recovered in the presence of βAPN (Figure 2.13).

2.4 DISCUSSION

The purpose of the work described in this chapter was to analyse whether alginate beads provide a biochemical environment similar to that of cartilage for the culture of chick chondrocytes, and to assay the effect of βAPN in the culture system. Collagen II production is the accepted indicator of chondrocytic phenotype, whereas the expression of collagens I and III are specific markers of dedifferentiated cells (Cancedda *et al.,* 1995). It was found that collagen II is produced in large quantities and is present in all three compartments (CM, FRM, medium) of the culture system (Figure 2.3) while collagens I and III were not detected in any compartment. Collagens IX and XI, that each comprise 10% of total collagen in cartilage, were also shown to be present (Vaughan *et al.*, 1988). These observations imply that in alginate beads chondrocytes remain phenotypically stable and produce collagens identical to those of cartilage.

Collagen IX recovered from the medium is present in both proteoglycan and non-proteoglycan forms, distinguishable by the presence/absence of a chondroitin sulphate GAG side-chain, respectively (Figure 2.6). The reason for production of two different forms of collagen IX is unclear but due to the location of collagen IX on the surface of collagen II fibrils, the GAG chain may regulate interactions of the collagen network with cell surfaces or other matrix components (Vaughan *et al.,* 1988). It has been shown that the chondroitin sulphate chain is one of the potential sites through which proteoglycans interact with other matrix components (Yamagata *et al.*, 1989) and thus its presence on collagen IX implies a regulatory role for this collagen type in the ECM (Bruckner *et al.,* 1988). Alternatively, the GAG chain may be involved in guiding collagen IX to the correct docking site on the collagen II fibril where it is subsequently cleaved to leave the non-proteoglycan form of the molecule (section

1.3.3.3; Diab *et al.,* 1996). Both forms of collagen IX have been shown to be present in embryonic chick cartilage (Yada *et al.,* 1992) and thus the finding that chondrocytes cultured in alginate beads produce both forms suggests once again that this culture system provides an environment similar to that of cartilage.

The 2 forms of collagen IX co-exist *in vivo* but the amount of each form depends on the tissue source: 80% of collagen IX extracted from embryonic chicken cartilage is present as the proteoglycan form whereas only 5% of collagen IX extracted from bovine articular cartilage is in the proteoglycan form (Huber *et al.,* 1988; Ayad *et al.,* 1989; Yada *et al.,* 1992). It was found that 65% of collagen IX produced in alginate bead culture is present in the proteoglycan form. The reason for the slight reduction in the proteoglycan form produced here compared to that *in vivo* is unclear but there may be losses in the DEAE-Sephacel chromatography step or, due to the high negative charge on the alginate, less of the proteoglycan form of collagen IX is produced.

The high concentration of collagen IX recovered from the medium suggests that this collagen type interacts relatively weakly with other components of the matrix. This, however can be explained by the absence of fibrils in the matrix, which means there was no surface to which collagen IX molecules could attach and thus their high solubility in the medium (see Chapter 3).

Collagen XI was present in unusually small quantities in the CM (3%) when culturing in the absence of βAPN (Table 2.1), but pepsin digestion and SDS extraction of the insoluble pellet that remained after neutral salt-extraction of the CM showed the presence of collagen XI (11%; Figure 2.11). These results indicate that collagen XI is indeed an integral part of the CM compartment and is present in concentrations comparable to that of cartilage. Collagens IX and X were also found in the insoluble pellet although they were not detected in the 1M NaCl supernatant, even in the presence of βAPN (Figure 2.11). Collagen X is also recovered from the crude extract of the CM (Figure 2.2) although it was absent in other results (Figures 2.3 and 2.9). This implies that the partial purification methods employed in this study results in the loss of some collagen types. As collagen X was detected after minimal

purification (an ethanol precipitation) and collagens IX and X were present following a highly efficient extraction (SDS), it can be concluded that further purification steps or less efficient extraction (1M NaCl) led to the loss of collagens IX and X.

The chondrocytes cultured in alginate beads were derived from the whole sterna and therefore represent all the zones of differentiation found *in vivo*, that is, the superficial, the resting, the proliferative and the hypertrophic (Figure 1.3). Collagen X is a specific marker of hypertrophic chondrocytes and its presence in this culture system implies that alginate beads allow chondrocytes to remain in the same stage of differentiation as when isolated from cartilage, as has been shown in other suspension cultures where chondrocytes show features corresponding to the depth of cartilage from which they came (Schenk *et al*, 1986; Aydelotte *et al.*, 1988).

As mentioned previously (section 2.1) there have been 2 other studies analysing collagen production in the alginate bead culture system but these pertain to chondrocytes derived from rabbit or bovine articular cartilages (Ramdi *et al.*, 1993; Petit *et al.*, 1996). The results of each study show differences to each other and also to the results presented here. For example, collagen synthesis by bovine chondrocytes increased steadily over a 28 day culture period and that produced by chick chondrocytes increased over a 7 day culture period (Figure 2.5) whereas collagen synthesis by rabbit chondrocytes plateaued after 4 days in culture. These contrasting results confirm that ECM's differ depending on the species from which the chondrocytes were derived (Elima and Vuorio, 1989). There was one major difference between the results presented here and that of Petit *et al.*, 1996 in that relatively small amounts of collagens were detected in the medium of cultured bovine chondrocytes compared to the large amounts of collagens II and IX recovered in this study. Again this may be due to variations in the ECMs of different species. There were also similarities between the studies: the ratio of collagen types produced by both chick and bovine chondrocytes were similar to those *in vivo,* and both these studies found that more collagen was recovered from the CM than the FRM.

2.4.1 Effect of βAPN

Traditionally, βAPN is added to all the culture systems to increase solubility of collagen and hence aid its recovery (Steinmann *et al.*, 1993). The results presented here show that collagens II and XI were more readily extractable from the CM in the presence of βAPN (Figure 2.11). Although cell numbers were unaffected (Figure 2.8), total collagen production was reduced by 50% in the presence of βAPN (Figure 2.5), which is in agreement with studies *in vivo* (Kato *et al.*, 1995). This suggests that the addition of βAPN to the medium is not toxic to the cells but may selectively decrease the production of cartilage collagens. Alternatively, βAPN may induce chondrocyte dedifferentiation to the resting state where relatively small amounts of collagens are produced (Figure 1.3). This is corroborated by the fact that collagen X, a specific marker of hypertrophic chondrocytes, is not detected in the CM in the presence of βAPN (Figure 2.11). Conversely, in the absence of βAPN, chondrocytes may be in the proliferative and/or hypertrophic states of differentiation where there is high production of cartilage-specific collagens (see Chapter 3) and collagen X is detected when culturing in the absence of βAPN (Figure 2.11).

There was a steady increase in the amount of proteoglycan recovered from the medium during the 14 day culture period regardless of the presence of βAPN. In these cultures, as in cartilage, the organisation of the collagen network plays an important role in trapping PG-aggregates (Kimura and Kuettner, 1986). The steady increase of proteoglycan found in the medium may be due to the lack of a fibrous collagen network to trap proteoglycan aggregates (see Chapter 3) and the proteoglycans in the medium are therefore likely to represent either non-aggregated proteoglycans or subunits/fragments of proteoglycan molecules, especially aggrecan. The increasing amount of proteoglycan in the medium also represents the high turnover rate of proteoglycans in alginate culture (Mok *et al.*, 1994; Hauselmann *et al.*, 1992). Alternatively, as hyaluronic acid is required to aggregate and stabilise the proteoglycan aggregates (Aydelotte and Kuettner, 1993) and as it is negatively charged, the charge repulsion between the hyaluronic acid and the alginate may force this glycosaminoglycan to be discharged into the medium and thus it would not be

available for aggregating the proteoglycans in the CM. The affects of charge could be directly investigated by culturing chondrocytes in a neutral suspension e.g. agarose.

In the presence of βAPN there was an increase in the total proteoglycan (CM/FRM + medium) production but in the CM/FRM the concentrations decreased (Figures 2.13 and 2.12). The inhibition of cross-link formation has already been shown to decrease total collagen production (Figure 2.5) and thus proteoglycans recovered from the CM/FRM have less collagen in which to become entrapped, resulting in the increased solubility in the medium. The increase in total proteoglycan is more difficult to explain, but a decrease in collagen production may trigger an increase in proteoglycan synthesis in an effort to form a functional matrix.

2.4.2 Final Conclusions

In conclusion, several lines of evidence have shown that alginate beads provide an environment like that of cartilage for the culture of chick chondrocytes. The observation that the cartilage-specific collagens are produced in abundance indicates that chondrocytes remain phenotypically stable in alginate beads. The ratios of the collagen types present, the appearance of collagen IX in both the non-proteoglycan and the proteoglycan form and the production of proteoglycans, also point towards this system being similar to that *in vivo*.

Although the above results indicate that some properties of alginate beads are similar to cartilage, not all the results observed confirmed this. For example, a large proportion of collagen II synthesised retained the N-propeptide (chapter 4) while the total amount of collagen IX recovered was much greater than that present *in vivo*. These differences may be a direct result of the alginate bead culture system as the chondrocytes may modify their phenotype and thus synthesise different/different ratios of ECM molecules due to the abundance of certain growth factors in the medium, the ready supply of nutrients or due to the lack of mechanical forces that they are subject to *in vivo*.

Chapter 3

Structure Of ECM Produced By Chondrocytes Cultured In Alginate Beads

3.1 INTRODUCTION

Chondrocytes derived from articular cartilage show characteristics that are dependent on many factors, including depth/position within cartilage, cell density and the age of the organism (section 1.3.2; Grandolfo *et al.*, 1993). For example, chondrocytes derived from the resting zone are small, show little mitotic activity and synthesise small quantities of cartilage macromolecules while chondrocytes of the proliferative zone have high mitotic activity and secrete large amounts of the cartilage-specific collagens (Tschan *et al.*, 1993). The features shown by individual chondrocytes *in vivo* are retained when the cells are subsequently isolated and cultured in suspension *in vitro* (Aydelotte *et al.*, 1986).

The ECM that surrounds chondrocytes in each zone can be subdivided into several regions known as the pericellular, the territorial and the interterritorial matrices (Figure 3.1; Mok *et al.*, 1994). The pericellular matrix is very thin and is characterised by the absence of cross-banded fibrillar collagens although it is rich in proteoglycans. Conversely, the territorial matrix is composed of a fine network of fibrillar collagens that surrounds both individual chondrocytes and those present in chondrons (Mok *et al.*, 1994; section 1.3.2). The interterritorial matrix is the furthest region from the cell and here the collagen fibrils run in parallel and are interspersed by proteoglycans. The different compartments of alginate bead culture represent these different matrices; the CM represents the combined pericellular and territorial matrices while the FRM depicts the interterritorial matrix (Hauselmann *et al.*, 1994).

Previously, it was suggested that procollagen secreted in high concentrations from cells in culture formed specific aggregates called segment-long-spacing crystallites (SLS; Hulmes *et al.*, 1983). In each aggregate, collagen molecules are arranged side by side with their NH_2- and COOH-terminal ends in register (Gross *et al.*, 1954; Kielty *et al.*, 1984). SLS-type aggregates with collagen-like banding patterns have been reported to be present both within cells and in the pericellular environment *in vivo* (Perez-Tamayo, 1972; Warshawsky, 1972; Weinstock and Lebond, 1974; Weinstock, 1977; Fernandez-Madrid *et al.*, 1980).

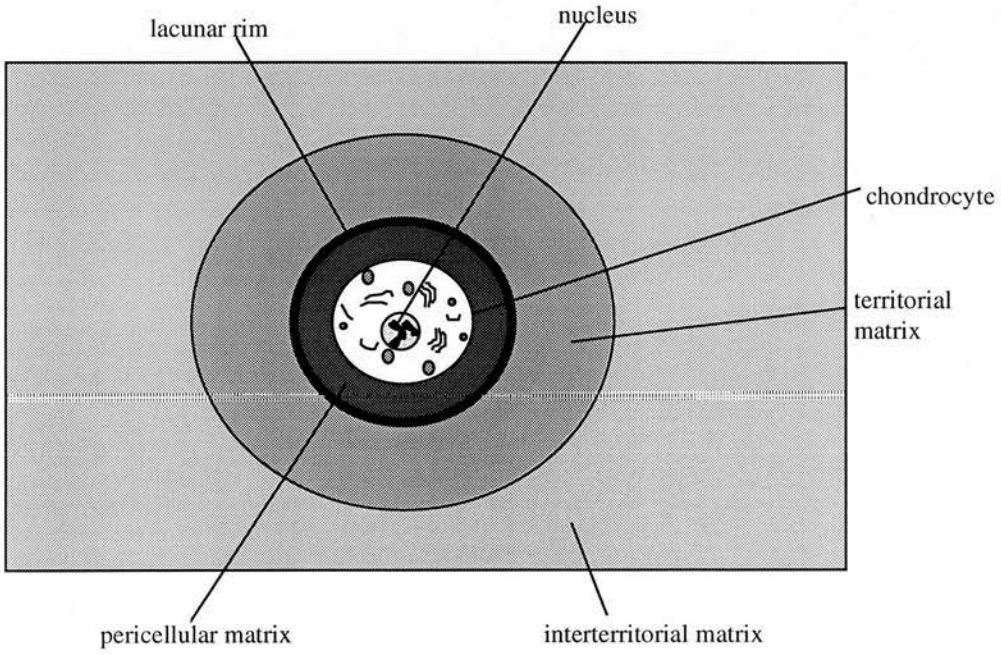


Figure 3.1. Idealised chondrocyte with surrounding matrices. In alginate culture, the CM represents the combined pericellular and territorial matrices while the FRM represents the interterritorial matrix. (Adapted from Horton, 1993).

This chapter analyses the structure of the ECM produced by chick chondrocytes in each compartment of alginate bead culture and compares it to that of cartilage. More specifically, what type of assembly does the newly synthesised collagen form?. Light microscopy and TEM show the morphology of the chondrocytes throughout the 14 day culture period while TEM analysis reveals the structure of the newly secreted collagen. The major observation was that SLS aggregates were found instead of banded collagen fibrils in both the territorial and interterritorial matrices, suggesting that alginate inhibited the formation of collagen fibrils. This was investigated by reconstituting collagen I fibrils *in vitro* in the presence of alginate.

3.2 Material and Methods

3.2.1 Materials

Four hundred mesh, formvar/carbon-coated copper grids, gold grids (400 mesh), glutaraldehyde (electron microscopy grade), Agar 100 resin, osmium tetroxide, polypropylene oxide, BEEM capsules and uranyl acetate were from Agar, Stansted, Essex; PBS was from Gibco Life technologies, Paisley, Scotland unless stated differently in the text; collagen I was from Coletica (courtesy of Dr. D. Herbage); poly-L-lysine and Tmax 400 film was from Sigma, Poole, Dorset; fluoroscein isothiocyanate (FITC) conjugated and rhodamine conjugated goat anti-rabbit antibodies were from the Scottish Antibody Production Unit (SAPU), Carluke, Lanarkshire. All other reagents (analytical grade) were from BDH, Poole, Dorset unless stated differently in the text.

3.2.2 Light Microscopy of Cells in Alginate Beads

On day 14 of the culture period, whole beads were taken out of culture and placed on a glass microscope slide in a drop of PBS (Gibco). The slide was placed on the stage of a Zeiss Axioplan microscope and viewed using phase contrast optics. Photographs were taken using Ilford IP4 film.

3.2.3 Embedding Cells for Analysis by TEM

On the days stated in the text, alginate beads were embedded for morphological analysis. In all subsequent procedures, 10mM $CaCl_2$ was present to maintain polymerisation of the beads. Whole beads were fixed with 2% glutaraldehyde in PBS for 1 hour at room temperature. Samples were washed thoroughly in PBS and post-fixed in 1% (w/v) osmium tetroxide in PBS (1 hour at room temperature). After washing in dH_2O and dehydration through a series of graded absolute alcohol's (10 minutes in each of the following: 30%, 50%, 70%, 80%, 95%, 100%, followed by 1x10 mins in 1:1 polypropylene oxide (PO):100% ethanol, 2x5 mins in PO and 1x10 min in PO), the samples were embedded in Agar 100 resin, an Epon 812 substitute, as follows. PO and Agar 100 were mixed 1:1 and added to each sample for 1 hour with rotation. Fresh Agar 100 was added overnight, with rotation and for 4 hours the next day. Finally, the samples were placed at the point of a BEEM capsule and fresh Agar 100 was added and placed at 60 °C for 24 hours until the resin was fully polymerised. Ultrathin sections were then cut using a ultramicrotome (LKB) and picked up on gold grids (400 mesh). The sections were contrasted with 1% (w/v) aqueous uranyl acetate, pH 4.4 and 0.1% lead citrate, pH 12 before viewing by TEM (Philips CM12 electron microscope).

3.2.4 Analysis of SLS Aggregates in Alginate Bead Culture

On the days stated in the text, alginate was solubilised with 55mM sodium citrate and the cells with their associated matrix were collected by centrifugation as described previously (section 2.2.2). The cell pellet was resuspended by gentle agitation in 0.5ml PBS and the suspension was poured onto Nescofilm. Formvar/carbon-coated copper grids (400 mesh) were floated on the suspension for 10 mins and then removed, blotted with filter paper and allowed to air-dry at room temperature. Each grid was then negatively stained for 1 min with 1% (w/v) phosphotungstic acid, pH 7.3 and the absorbed structures were observed by TEM (Philips CM12 electron microscope).

3.2.5 *In Vitro* Fibrillogenesis in the Presence of Alginate

Collagen I (0.5% (w/v) in 0.5M acetic acid) was dialysed against 5mM acetic acid and diluted to a concentration of 1mg/ml with 5mM acetic acid. This solution of collagen I monomers was then mixed 1:1 in a cuvette at 0 °C with either 2% alginate (in 2xPBS) or 2xPBS (46.4mM Na_2HPO_4, 13.6mM NaH_2PO_4, 135mM NaCl, pH 7.4; Williams *et al.*, 1978). To initiate fibril formation, the mixtures were warmed to 37 °C in a spectrophotometer (Philips/Unicam 8700 series UV/VIS) that had previously been "blanked" on a 1:1 mixture of 5mM acetic acid and 2xPBS. The turbidity of each cuvette was continuously monitored for 6 hours at 313 nm with readings taken every 5 mins. The kinetic data was transferred to Microsoft Excel using Unicam ASDS software.

The resulting collagen suspension was poured onto Nescofilm and formvar/carbon-coated copper grids (400 mesh) were floated on the gels for 2 mins. The grids were then stained for 1 min with 1% (w/v) phosphotungstic acid, pH 7.3 before observation by TEM (Philips CM12 electron microscope).

3.2.6 Immunofluorescence

Chondrocytes were cultured for 7 days in alginate beads and the cells separated from the alginate by the addition of 55mM sodium citrate as described previously (section 2.2.2). The cell pellet was resuspended and washed twice in PBS before the cells were spun down onto coverslips that had previously been coated with poly-L-lysine. The cells were immediately fixed by addition of 3% (w/v) formaldehyde in PBS for 20 mins. All the following procedures were carried out at room temperature, ensuring the coverslips did not dry out at any time. Each of the washing steps were carried out over a period of 5 mins.

Following 3x2ml washes in PBS, 50mM ammonium chloride in PBS was added to the coverslips for 10 minutes to quench any remaining reactive groups of the formaldehyde. The coverslips were washed with PBS (3x2ml) followed by addition of 0.1% (w/v) Triton X-100 for 4 mins. Following further washing (3x2ml PBS,

3x2ml 0.2% gelatin/PBS), each coverslip was floated cell-side down for 20 mins on 50µl of primary antibody (affinity purified and diluted 1:100 with 0.2% gelatin/PBS). Coverslips were washed with PBS (3x2ml) and 0.2% gelatin/PBS (3x2ml) and then floated for 20 mins on 50µl of secondary antibody (either rhodamine or FITC conjugated goat anti-rabbit antibodies, diluted 1:100 in 0.2% gelatin/PBS). Coverslips were finally washed with PBS (3x2ml) and 0.2% gelatin/PBS (3x2ml) and mounted on a glass slide by dipping them in dH_2O, draining excess fluid on a piece of filter paper and then placing them cell-side down on a 15µl drop of Moviol. The Moviol was then left to harden overnight at 4 °C.

All slides were viewed using a fluorescent microscope (Leitz Ortholux 2) and photographs were taken using Tmax 400 film. Identical exposure times, both here (achieved by use of a Leitz camera controller) and at the printing stage, were adopted so that direct comparisons of the micrographs could be made.

For double labelling experiments, the above protocol was repeated from the primary antibody stage with collagen II antibodies being labelled with FITC-conjugated secondary antibody and collagen IX antibodies labelled with rhodamine-conjugated secondary antibody. When using FITC-conjugated antibodies, 0.5% n-propyl gallate (anti-fade) was added to the Moviol. Where Hoescht dye (33258) was used, 2 µg/ml (in PBS) was added to the coverslips and left to stain for 10 mins before washing and mounting onto glass slides. Hoescht dye was only used in conjunction with the rhodamine-conjugated secondary antibody.

3.3 RESULTS

3.3.1 Chondrocyte Morphology

Chondrocytes cultured in alginate beads *in situ* were analysed and their characteristics, for example, size and shape, compared to that of chondrocytes in all zones of cartilage. Also, the effect of βAPN on chondrocyte differentiation was investigated.

3.3.1.1 Light Microscopy of Chondrocytes in Alginate Beads

Whole alginate beads were placed on glass slides and the phenotype of the chondrocytes was analysed by light microscopy. It was found that the chondrocytes remained differentiated, that is, they retained their spherical shape over a 14 day culture period (Figure 3.2). Contrary to previous reports (Hauselmann *et al.*, 1994; Petit *et al.*, 1996), there didn't appear to be any flattened cells at the edges of the bead, which would indicate dedifferentiation. Chondrocytes *in vivo* are located within a chondron (section 1.3.2) where one or more cells are surrounded by the pericellular matrix and an enclosing capsule (Lee *et al.*, 1997) and although the cells are not in contact with one another, they remain viable. The chondrocytes in some regions of the alginate bead appeared to be in close association with one another which may indicate formation of a chondron (arrowheads, Figure 3.2).

3.3.1.2 Chondrocyte Morphology by TEM

Alginate beads were embedded in Agar 100, sectioned and viewed by TEM (Figure 3.3). In the chondrocyte shown, the most prominent organelle is the nucleus (N), with the more electron dense chromatin visible at its centre. Numerous mitochondria (M) are present throughout the cytosol which are characterised by their deep, invaginated cristae. The Golgi system (G) appears as flattened sacs and this organelle is known to make a significant contribution to the synthesis and the sulphation of GAG chains on proteoglycans (Stockwell, 1979). The rough endoplasmic reticulum (R) is not extensive in this particular chondrocyte but is most apparent in others (Figure 3.4d) and ribosomes are observed on the surface. Abundant rER and large amounts of Golgi vacuoles are indicative of hypertrophic chondrocytes (Bruckner *et al.*, 1989). Of the other organelles present, there is a large number of lysosomes (L) which contain many proteolytic enzymes that function in cell metabolism, turnover of

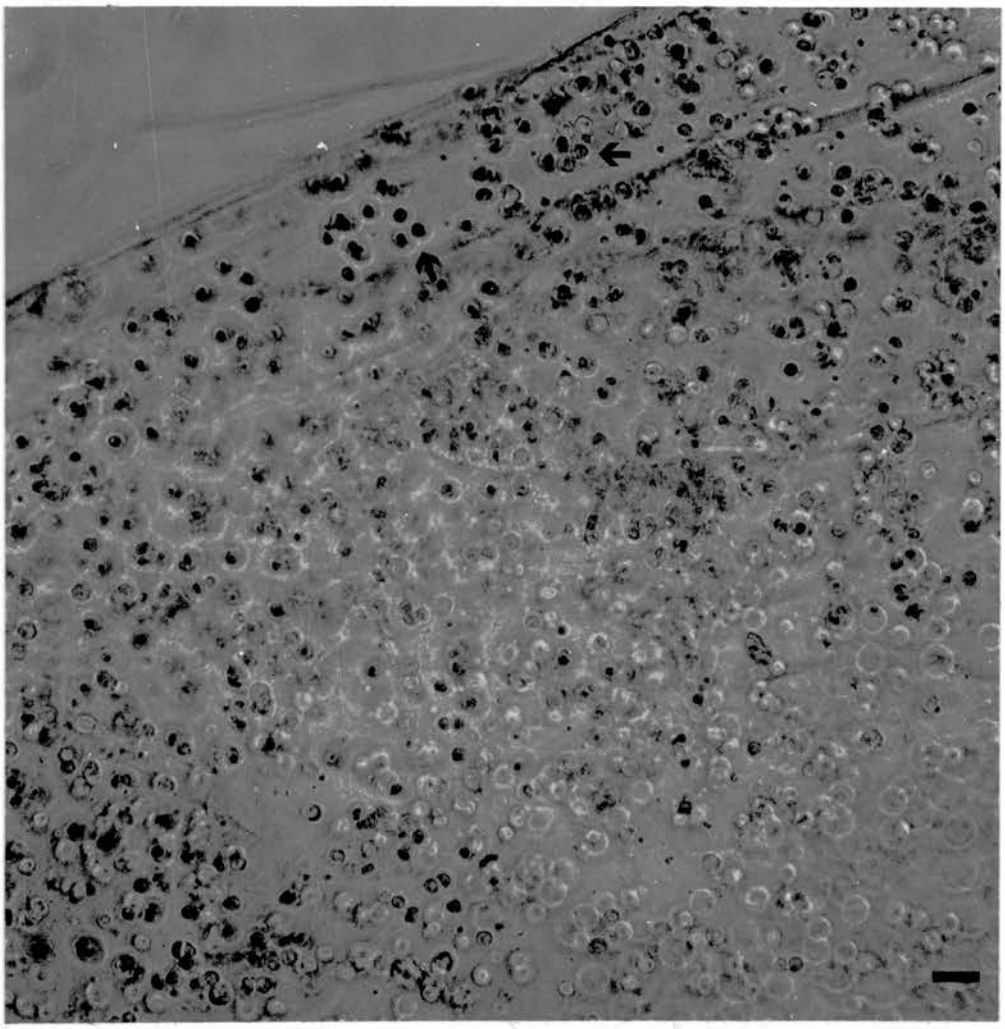


Figure 3.2. Light micrograph of chondrocytes cultured in alginate beads. Chondrocytes were found to be phenotypically stable over a 14 day culture period. No flattened cells were visible at the bead surface. Small arrow heads indicate cells in close association, probably within a chondron. Bar = 50μm.

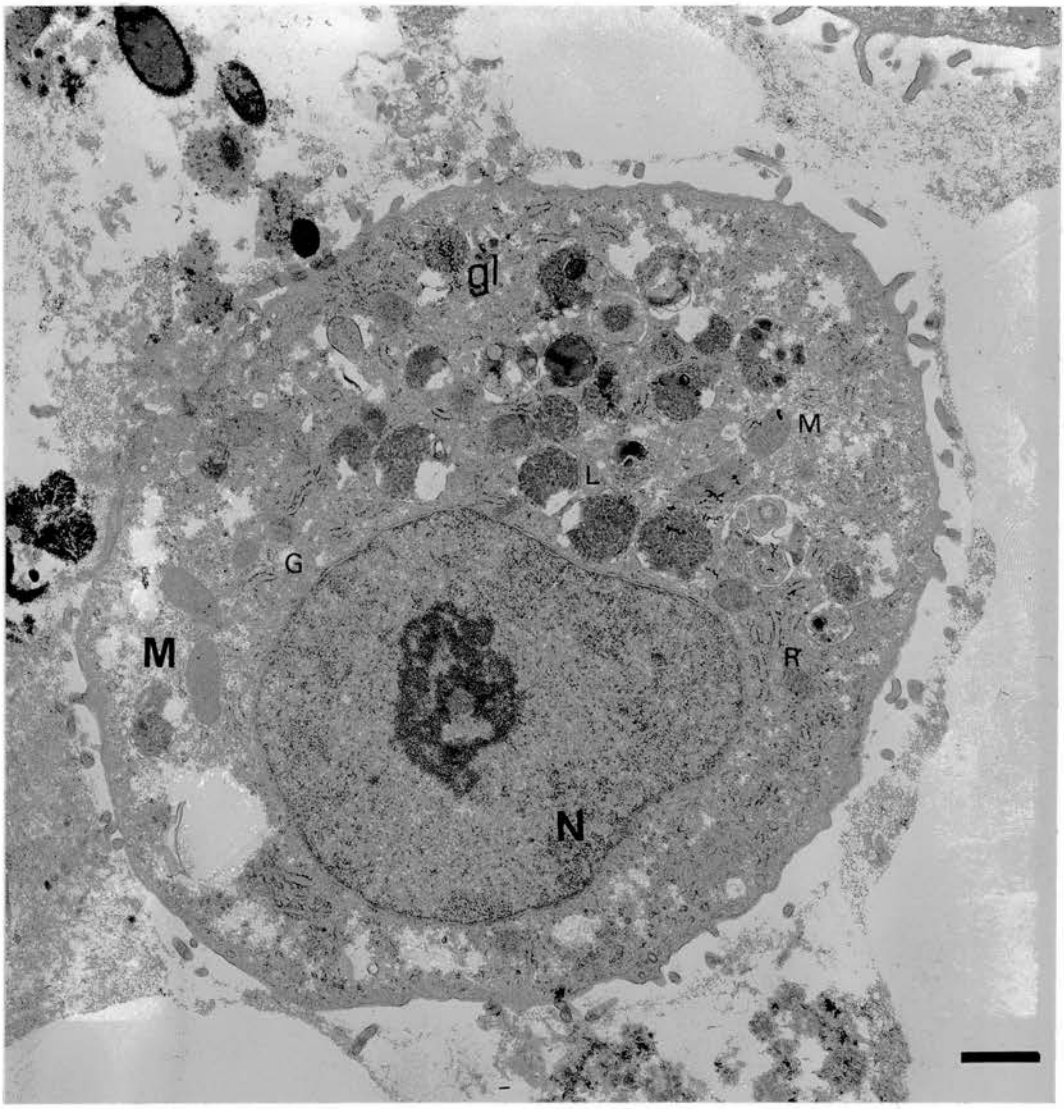


Figure 3.3. Electron micrograph of a typical chondrocyte from alginate bead culture. Whole beads were embedded, stained with osmium tetroxide and viewed by TEM. Organelles labelled are: nucleus (N), mitochondria (M), Golgi system (G), rough endoplasmic reticulum (R), lysosomes (L) and glycogen particles (gl). Bar = 2μm.

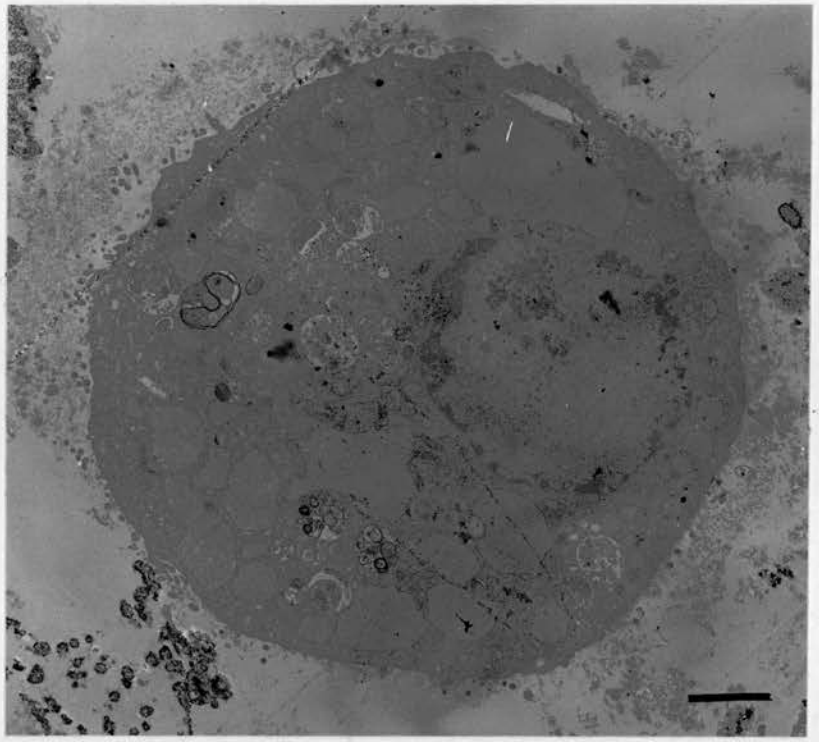
cytosolic organelles and local control of the pericellular matrix. The smaller, electron dense granules are glycogen particles (gl) that are more numerous in resting cells (Bruckner *et al.*, 1989).

Electron micrographs confirm that chondrocytes remain phenotypically stable within the alginate bead environment although there is a certain degree of variation in cell shape (Figure 3.4a-d). This is not surprising as the chondrocytes were derived from whole sterna, and therefore represent every zone of articular cartilage and the variations in cell shape indicate that the chondrocytes retain the characteristics of the zone that they were derived from. Cell diameter was also shown to vary, from 12μm to 28μm (Figures 3.4 and 3.5) which is similar to the size of chondrocytes *in vivo* (10μm to 30μm; Stockwell, 1979). Based on observations of cell diameters and the distribution of organelles within each cell (Bruckner *et al.*, 1989), the zone of cartilage that the chondrocytes were derived from is proposed (Figure 3.4).

3.3.1.3 Effect of βAPN on Chondrocyte Differentiation

It was found previously that the presence of βAPN in the culture medium reduced total collagen production by 50% (section 2.3.1.2) and it was proposed that this may be due to βAPN affecting chondrocyte differentiation; chondrocytes from the resting zone are smaller and produce relatively little ECM whereas chondrocytes from the proliferative zone are larger and synthesise abundant matrix. Therefore the sizes of chondrocytes from both culture conditions (that is, in the presence and the absence of 0.2mM βAPN) were assessed. It was found that in the presence of βAPN chondrocytes had an average size of 18.48μm whereas the cells were larger (21.64μm) in the absence of βAPN (Figure 3.5). This suggests that the presence of βAPN in the culture medium affects chondrocyte size and thus infers an affect on differentiation.

(a)



(b)

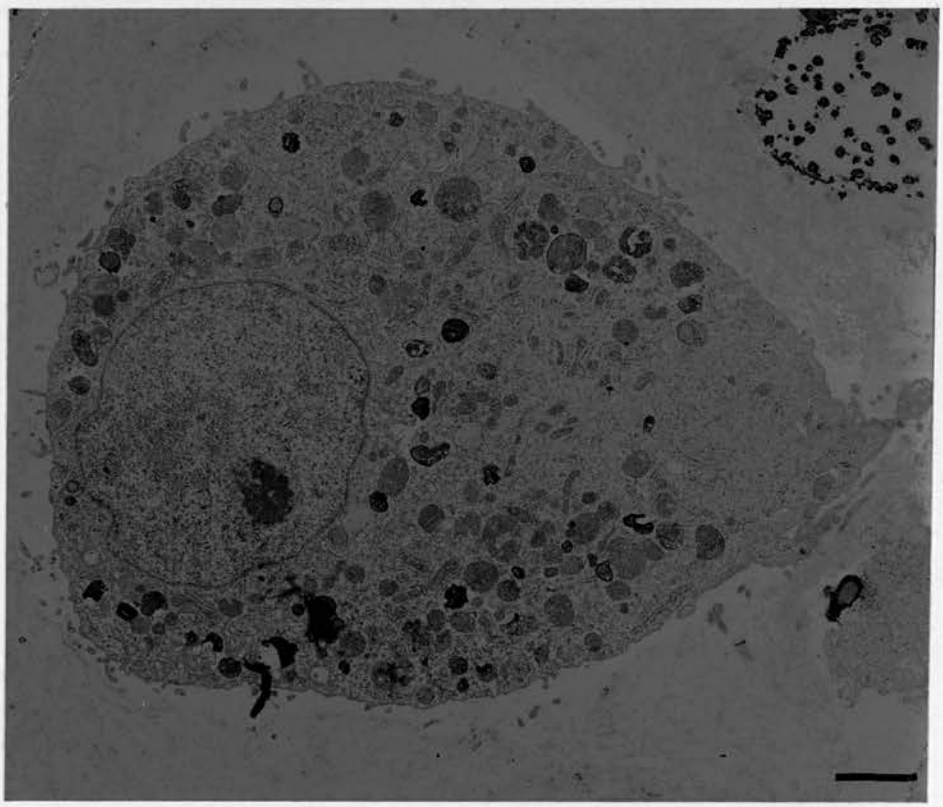
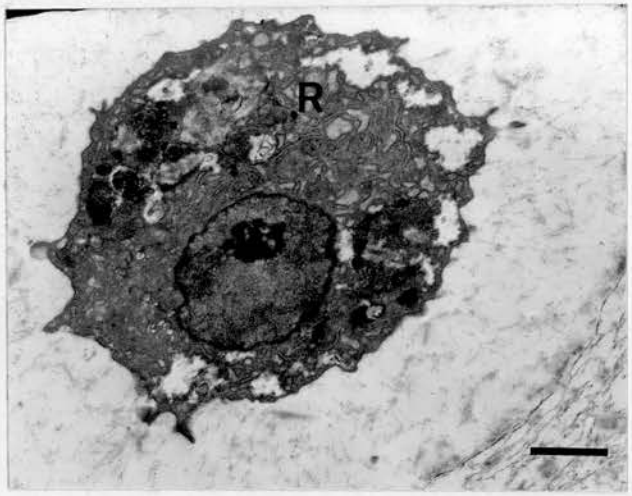
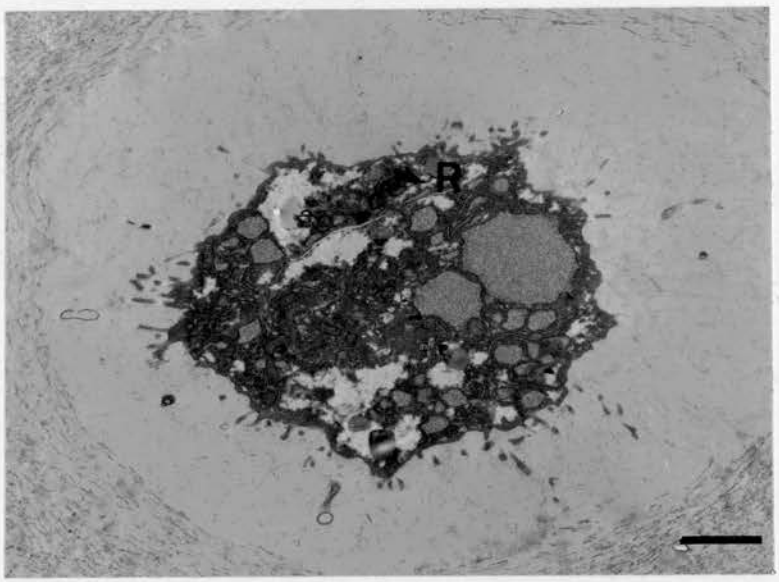


Figure 3.4. Variation in shape and size of chondrocytes in alginate bead culture. The appearance of cell organelles and chondrocyte sizes are summarised in the table and based on these observations the zone of cartilage from which each chondrocyte was derived is proposed. (a) and (b), bar = 2μm.

(c)

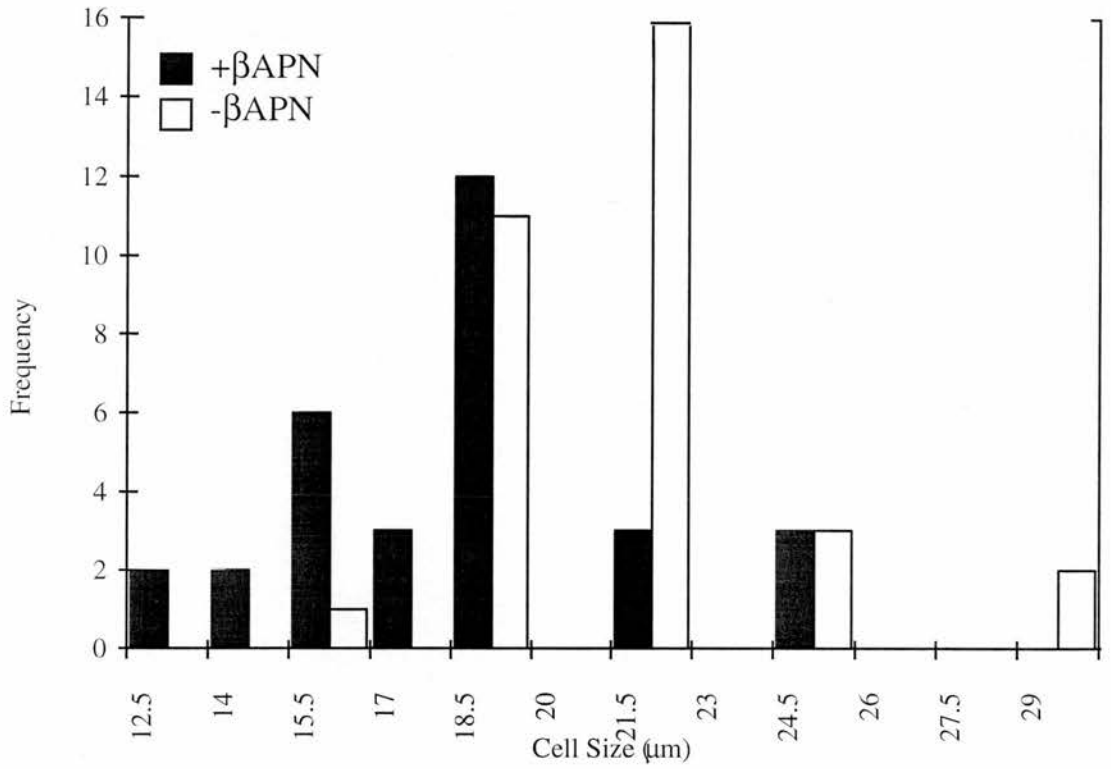


(d)



Cell	Size	N	M	G	rER	L	Proposed Zone
(a)	17μm	heterochromatic	✓	x	✓	✓✓✓	resting
(b)	18μm	euchromatic	✓✓✓	✓✓✓	✓✓✓	✓✓	proliferative
(c)	25μm	euchromatic	✓✓	✓✓	✓✓✓	✓	hypertrophic
(d)	26μm	euchromatic	✓✓	✓✓	✓✓✓	✓✓	hypertrophic

Figure 3.4. (Contd.) Nucleus (N), mitochondria (M), Golgi system (G), rough endoplasmic reticulum (R), lysosomes (L) and glycogen particles (gl). (c) and (d), bar =5μm.



+βAPN Average Cell Size = 18.48 μm ±0.61

-βAPN Average Cell Size = 21.64 μm ±0.46

Figure 3.5. Cell size when culturing in the presence and the absence of βAPN. Cells were measured from light micrograph negatives from day 14 cultures, either in the presence or absence of 0.2mM βAPN. Average cell size is shown with the standard error of the mean (n=31 for +βAPN, n=33 for -βAPN).

3.3.1.4 SLS Aggregates Replace Collagen Fibrils

A "halo" of matrix surrounds each cell, like that observed *in vivo*, although this "halo" contains few visible structures (Figure 3.6). Of the structures that were present, the cell membrane processes were the most apparent. The "halo" corresponds to the territorial matrix and thus fibrils were expected to be present with a classical cross-banded staining pattern. Fibrils were not apparent but SLS aggregates were observed (Figure 3.7; section 3.3.3). Other structures present, for example, amorphous material and electron-dense particles, probably pertain to the alginate gels.

Further away from the cell, at the edge of the "halo", an extensive, spindle-like network of alginate was visible (Figure 3.6). At the immediate edge of the "halo" of matrix, the alginate was aligned parallel to the cell membrane surface and it was in this area that the highest density of SLS aggregates were to be found (Figure 3.8).

3.3.2 Further Analysis of SLS Aggregates in Alginate Bead Culture

To investigate the structure of the SLS aggregates further, the CM was resuspended in PBS and copper grids were floated on this suspension. Again, no classical banded fibrils were observed but SLS aggregates were found to be present (Figure 3.9). The banding pattern of the SLS aggregate was unusual compared to that formed by procollagen I (Hulmes *et al.*, 1983; Koyabashi *et al.*, 1985). First, in all cases the banding pattern was centrosymmetric. In addition, the region surrounding the double light band at the centre of the aggregate had a wider diameter than regions on either side (Figure 3.9; small arrowheads) while both extremities were also wider (Figure 3.9; larger arrowheads). Amorphous black artefacts that surround each SLS aggregate probably pertained to residual alginate present in the resuspended CM or could represent calcium phosphate crystals forming during the staining procedure. The SLS aggregates had an average length of 346nm ±6.26 (Figure 3.10), although the distribution ranged from 100nm to 600nm.

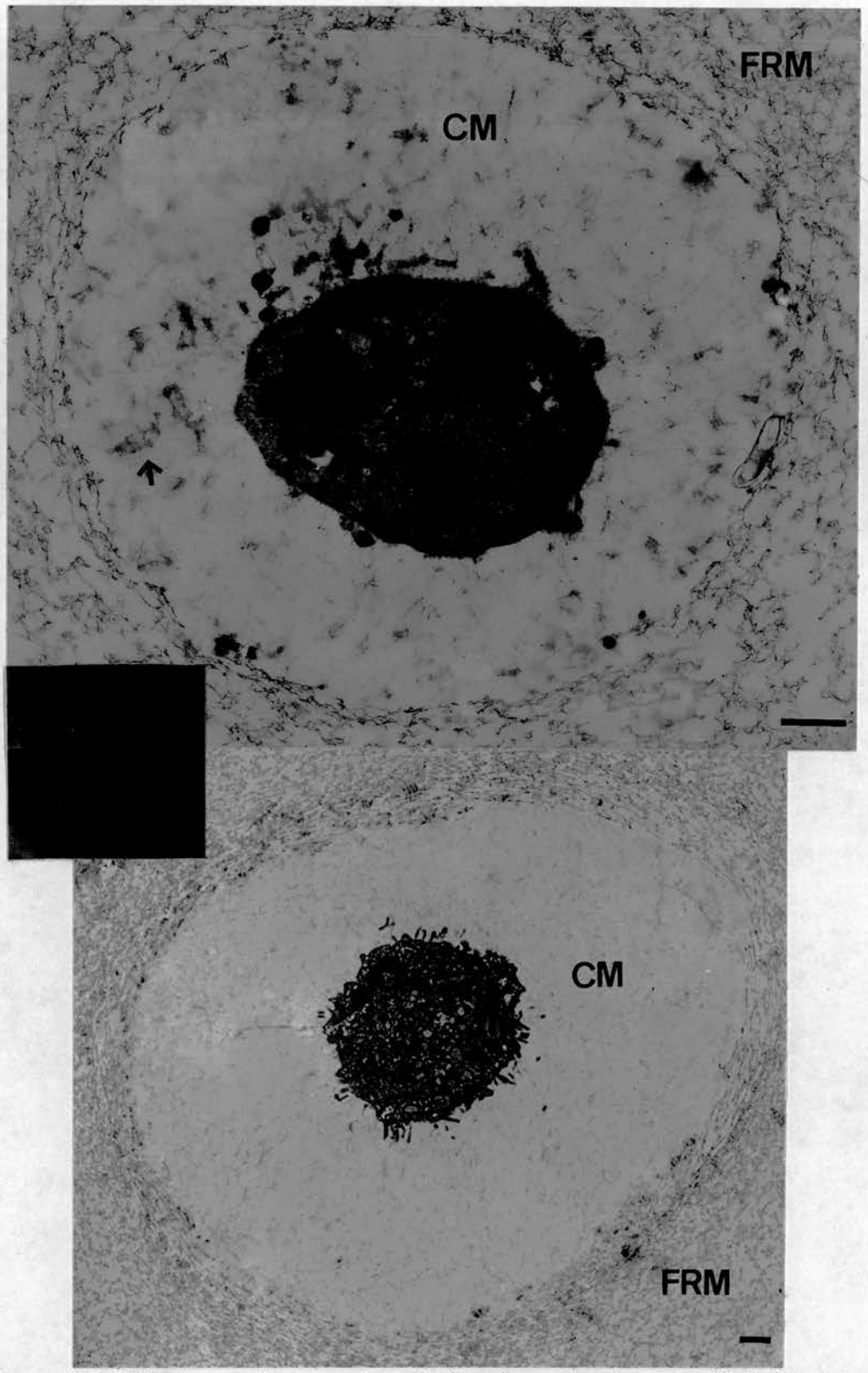
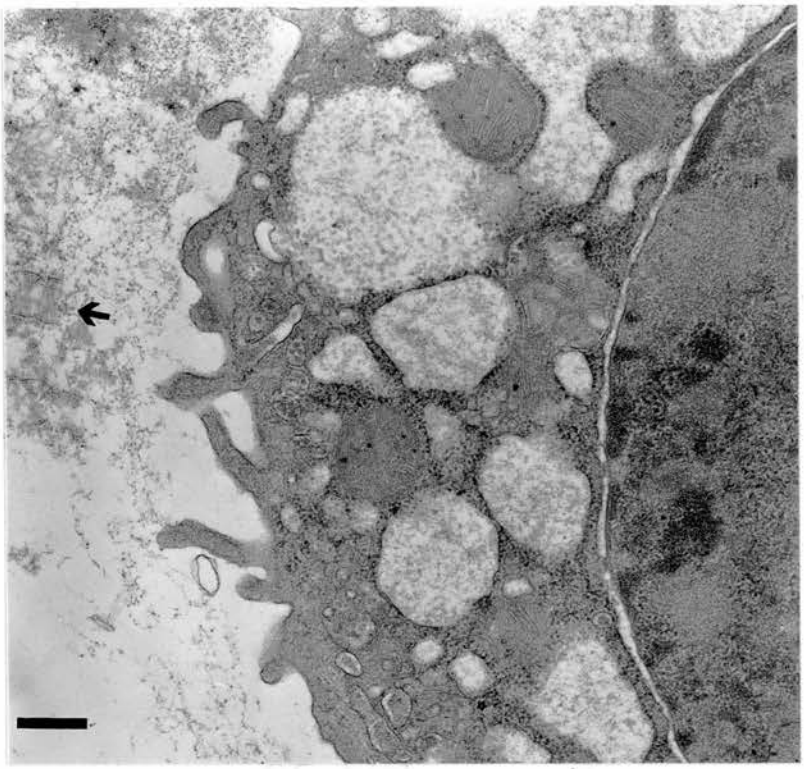


Figure 3.6. Electron micrographs of chondrocytes show a "halo" of matrix surrounding them. The CM and FRM compartments are indicated with the large arrows. The alginate forms a spindle-like network around the "halo", and is parallel to the cell membrane at the CM/FRM border. There are a few structures visible in the "halo" most of which pertain to the alginate gels, but also include SLS aggregates (small arrowhead). Inset shows spindle-like network of alginate formed without cells present. Bar = 4μm.

(a)



(b)

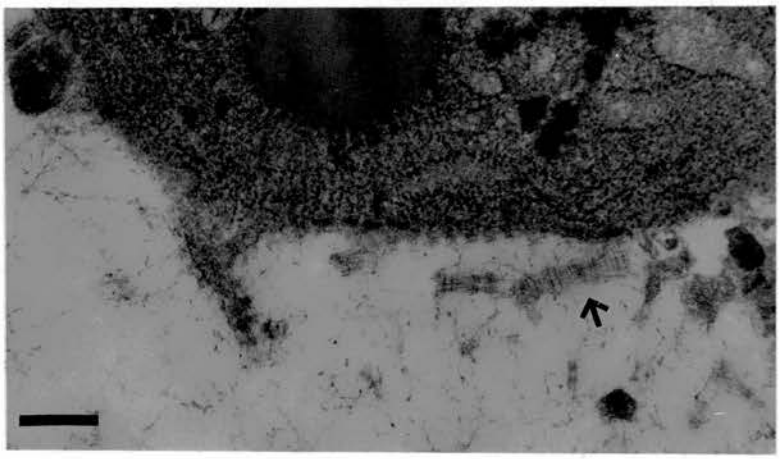


Figure 3.7. Electron micrographs showing SLS aggregates present in the CM. Arrowheads indicate SLS aggregates in close association with the chondrocyte. (a) bar = 500nm, (b) bar = 300nm.

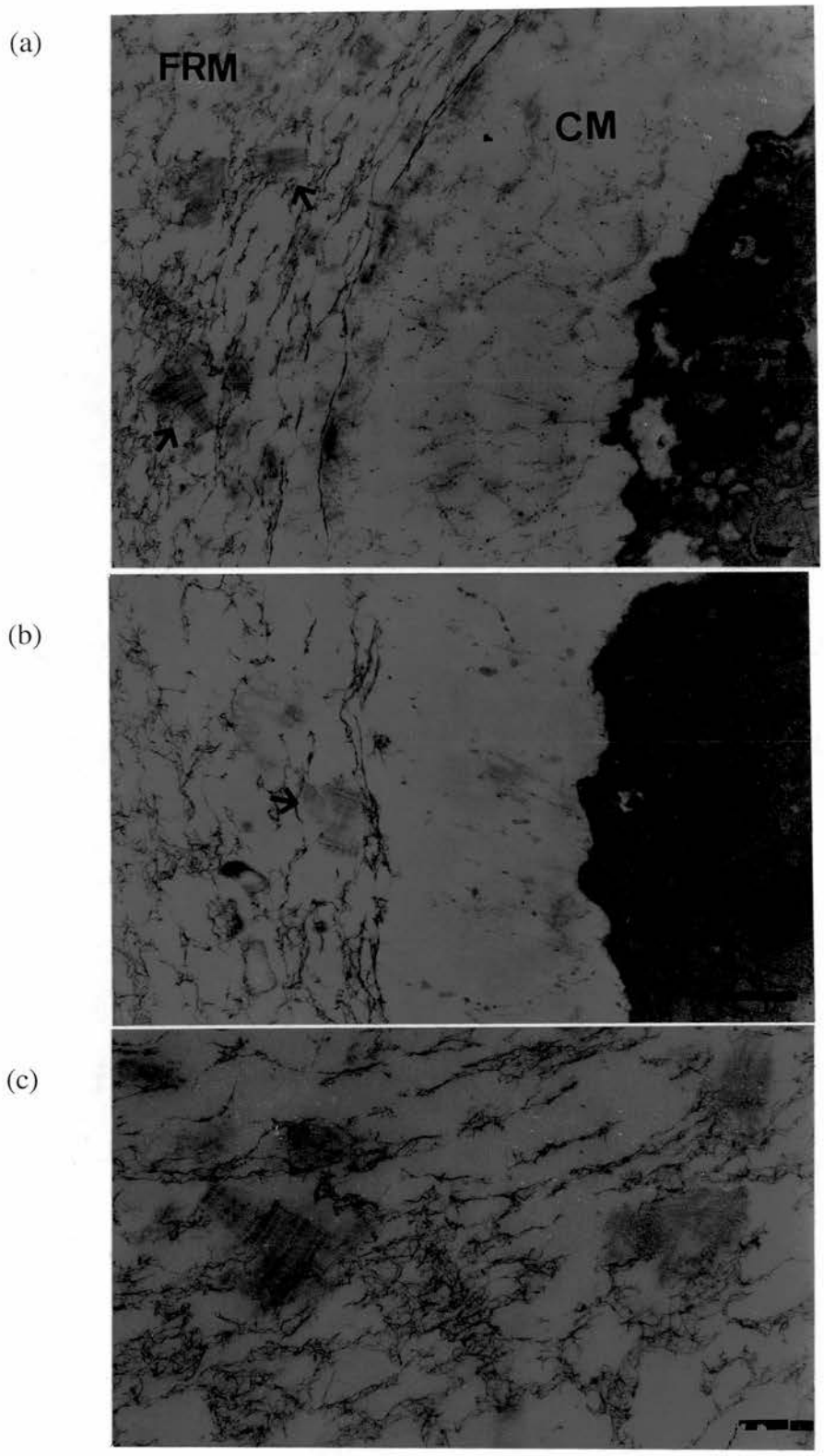


Figure 3.8. Electron micrographs showing SLS aggregates present in the FRM. SLS aggregates were present in a higher density at the edge of halo than any other region of the bead (a) and (b). (c) higher magnification of the CM/FRM border where most SLS aggregates were to be found. Arrowheads indicate SLS aggregates present in the FRM. (a) bar = 300nm, (b) and (c) bar = 400nm.

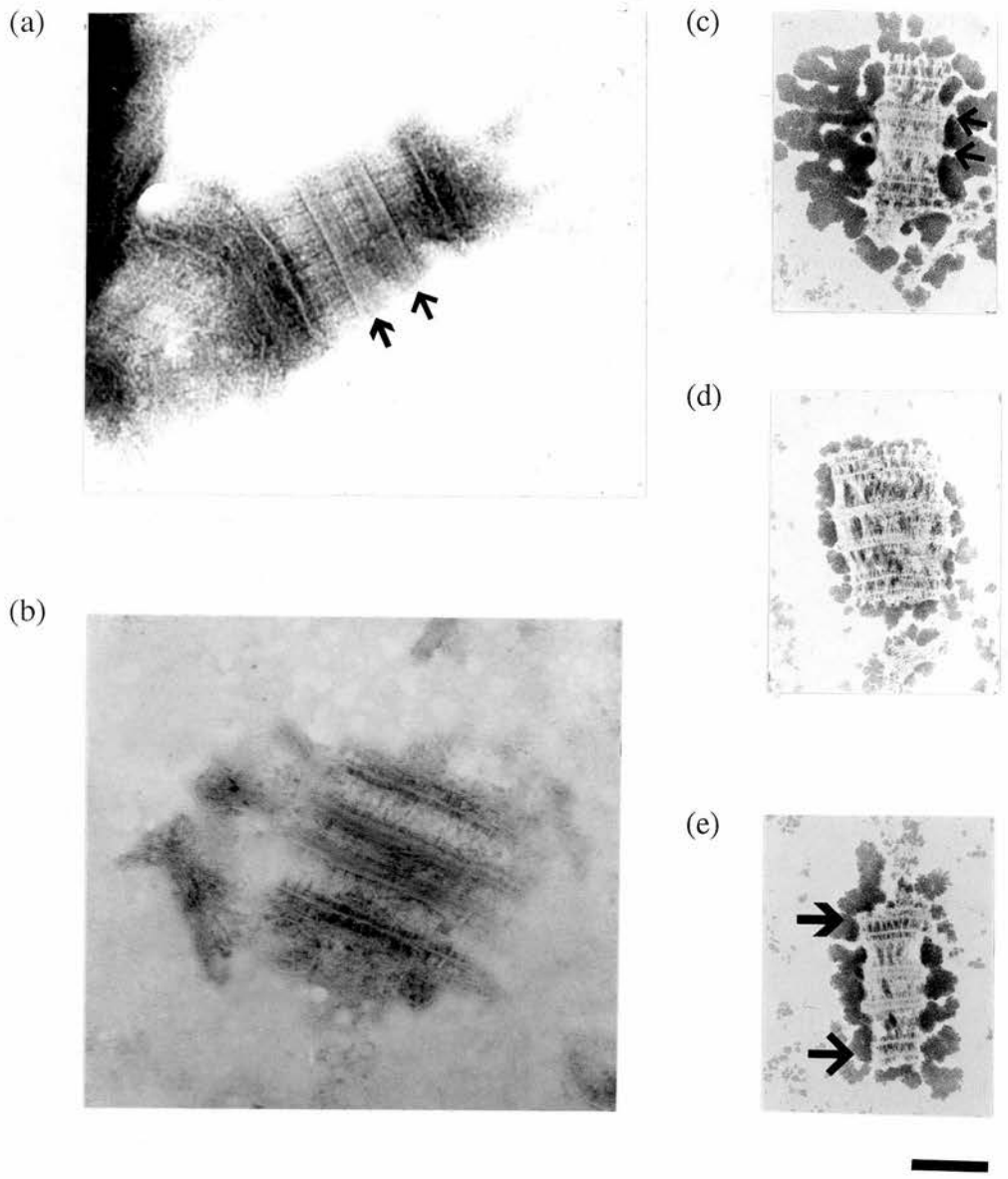


Figure 3.9. Segment-long-spacing crystallites (SLS) present in alginate bead culture. The CM was resuspended in PBS and copper grids were floated on the suspension for 10 mins. Following staining with 1% PTA, pH 7.4, grids were viewed by TEM. Amorphous black material surrounding each SLS aggregate probably pertains to residual alginate. (a) bar = 150nm, (b) bar = 100nm, (c), (d) and (e) bar = 140nm.

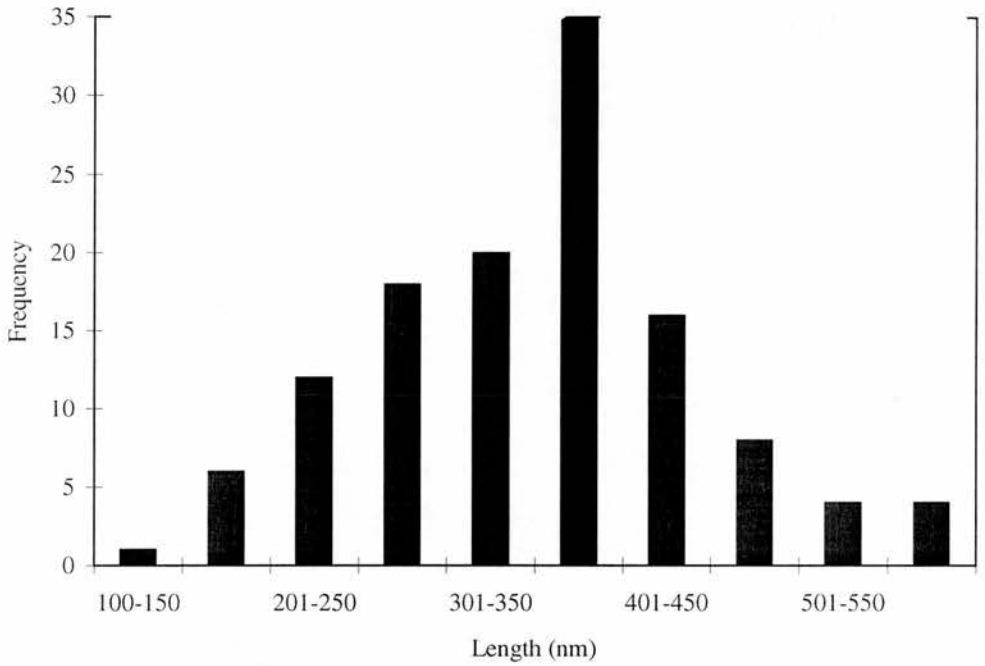


Figure 3.10. Measurement of the lengths of SLS aggregates. The CM was resuspended in PBS and copper grids were floated on the suspension for 10 mins. Following staining with 1% PTA, pH 7.4, grids were viewed by TEM and the lengths of SLS aggregates were measured from negatives. Lengths ranged from 100 to 600 nm, with an average length of 346nm ± 6.26 (standard error, n=124).

3.3.3 Effect of Alginate on Fibril Formation

The lack of collagen fibrils in this culture system suggested that alginate inhibited collagen fibril formation. To investigate this possibility, collagen I was reconstituted using the "cold-start" technique, in the presence or absence of 1% alginate. The "cold-start" technique requires the collagen solution and the double strength phosphate buffer to be kept at 4 °C prior to warming (Williams *et al.*, 1978). The acidic collagen solution is neutralised by the double strength buffer and fibril assembly is initiated by warming the mixture to 37 °C.

Analysis of turbidity showed that, in the absence of alginate, fibrils formed with a typical sigmoidal kinetic curve (Figure 3.11). Conversely, in the presence of alginate the turbidity did not increase but it showed a slight decrease over the time period. Considering that a solution of alginate is slightly turbid and that the spectrophotometer was "blanked" on a mixture of acetic acid and PBS, this would account for the higher absorbance observed in the presence of alginate.

When the contents of the cuvettes were absorbed onto copper grids and viewed by TEM, it was found that classical banded collagen I fibrils formed in the absence of alginate (Figure 3.12a). Conversely, in the presence of alginate, no banded fibrils were present but there was a high occurrence of rod-like structures (Figure 3.12b). No SLS aggregates were observed in either condition. This experiment would more adequately reflect the environment of the alginate bead if $CaCl_2$ was also present in the fibril formation buffer as this would stop any pH disparities that the non-gelled alginate may have.

3.3.4 Immunofluorescence

Chondrocytes were cultured for 14 days in alginate beads and the cells spun down onto coverslips. The ECM was analysed by immunofluorescence microscopy using affinity purified polyclonal antibodies to both collagens II and IX, with pre-immune serum substituting the antibodies as a control. It was found that antibodies to collagens II and IX labelled both the chondrocytes and the ECM (Figure 3.13).

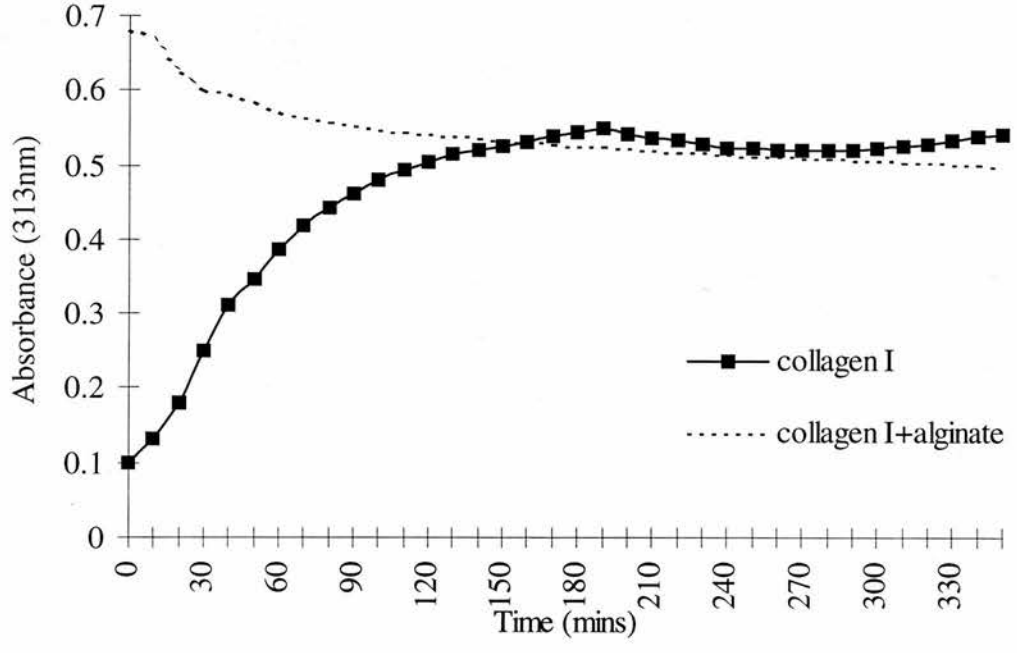


Figure 3.11. Alginate inhibits fibril formation *in vitro* .1. Kinetic data of collagen I fibrils reconstituted in the absence and the presence of 1% alginate. Collagen I (1mg/ml) samples were mixed 1:1 at 0 °C with either a 2% alginate in 2x PBS or 2x PBS. The mixtures were warmed to 37 °C to initiate fibril formation and turbidity was recorded at 313 nm.

(a)

(b)

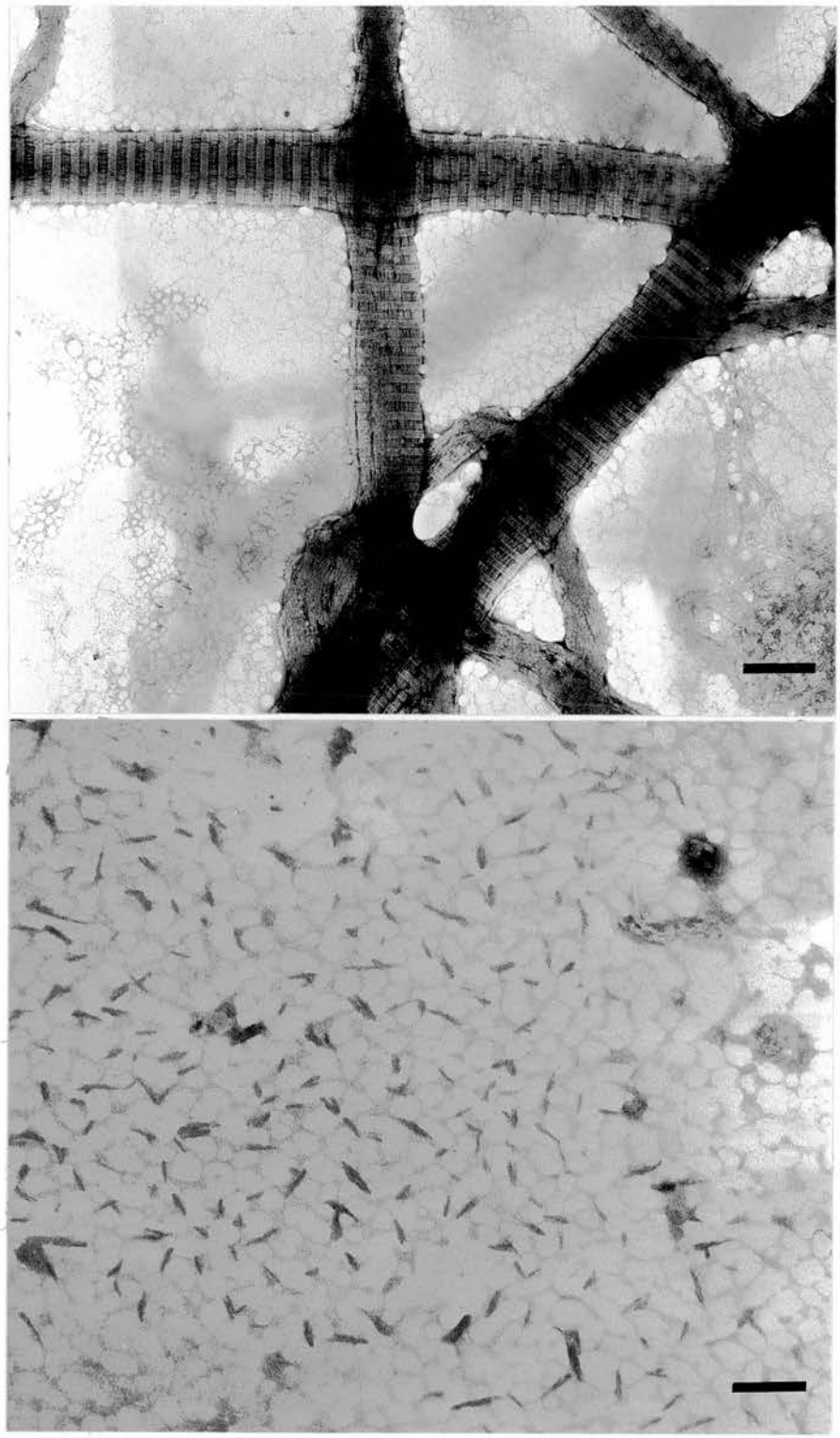


Figure 3.12. Alginate inhibits fibril formation *in vitro* .2. Fibrils reconstituted in the absence (a) and presence (b) of alginate were absorbed to copper grids and stained for 1 min with 1% phosphotungstic acid, pH 7.3 before observation by TEM. (a) bar = 100nm, (b) bar = 60nm.

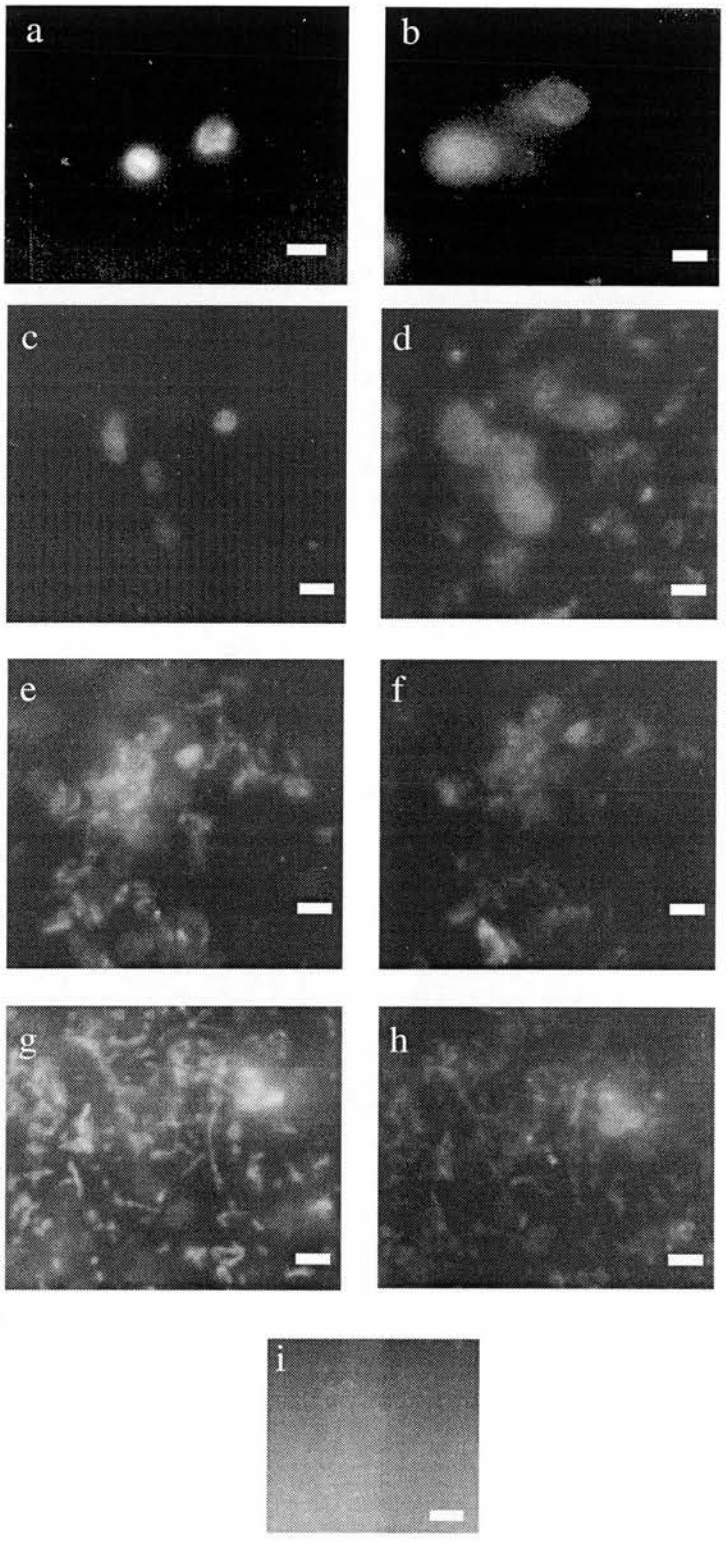


Figure 3.13. Immunofluorescence of chick chondrocytes after 14 days in culture. Cells were labelled with Hoescht dye (a and c) or affinity purified polyclonal antibodies (diluted 1:100) to either collagen IX (b) or collagen II (d). Double labelling experiments show co-localisation of collagens II and IX in the ECM (e and f, g and h). Substitution of primary antibody with pre-immune serum shows no labelling (i). Bar = 26 μm.

extracellularly (Figure 3.13e,f and g,h) and when pre-immune serum replaced the primary antibody there was no visible fluorescence (Figure 3.13I).

3.4 DISCUSSION

This chapter has analysed the morphology of ECM produced by chick chondrocytes cultured in alginate beads, at both light and electron microscopy levels. It was shown that the chondrocytes remained phenotypically stable over a 14 day culture period but that SLS aggregates substituted collagen fibrils in both the CM and FRM compartments. This indicated that the culture system provides a good model system for the study of chondrocyte differentiation but since alginate interrupts the normal assembly pattern of collagen, alginate beads are a poor model for investigations into cartilage morphogenesis. The following discussion examines both these points.

3.4.1 Chondrocyte Morphology

The chondrocytes in alginate bead culture were shown by light microscopy to be phenotypically stable and in close association with one another (Figure 3.2). These groupings may be indicative of chondrons, specialised micro-environments that surround one or more cells *in vivo* and are comprised of pericellular matrix (section 1.3.2; Lee *et al.*, 1997). Chondrocytes in culture are markedly heterogeneous in morphology, which is dependent on the different zones of cartilage from which they are isolated. In culture, the cells show features characteristic of the depth of cartilage from which they were derived (Schenk *et al.,* 1986) and it was found here that there were differences in the morphology of the chondrocytes, ranging from slightly flattened forms to star-like morphologies (Figure 3.4). This confirms that the chondrocytes retain characteristics of the zone of cartilage from which they were derived when they are subsequently isolated and cultured in alginate beads. Hypertrophic chondrocytes have been shown to become osteoblast-like (star-shaped) when cultured in monolayer in the presence of ascorbic acid (Descalzi-Cancedda *et*

chondrocytes retain characteristics of the zone of cartilage from which they were derived when they are subsequently isolated and cultured in alginate beads. Hypertrophic chondrocytes have been shown to become osteoblast-like (star-shaped) when cultured in monolayer in the presence of ascorbic acid (Descalzi-Cancedda *et al.*, 1992), so the star-like forms found here may represent hypertrophic chondrocytes.

Chondrocytes were surrounded by a "halo" of ECM (Figure 3.6). The lack of collagen fibrils and the small amount of SLS aggregates indicates that this "halo" was most likely to be comprised of proteoglycan aggregates. The high negative charge of these molecules would repel the polyanionic alginate thus giving rise to the observed "halo". The proteoglycans present in the FRM may contain less GAG chains, and therefore have less charge associated with them. Thus charge repulsion becomes less of a hindrance, allowing the alginate and associated ECM to form the spindle-like network that is observed (Figure 3.6).

There were several structures visible in the "halo" surrounding the chondrocyte. There were numerous cell processes that *in vivo* are continually extended and retracted and therefore may increase matrix turnover in the pericellular matrix (Stockwell, 1979). The amorphous material and the electron dense particles present in the "halo" probably pertain to residual alginate. There are, however a number of other possible explanations. Collagen X, for example, has been shown to form hexagonal lattices *in vitro* (Kwan *et al.*, 1991) and as this collagen type was found to be an integral part of the ECM of this compartment (Figure 2.2), collagen X may represent some of the other material present in the "halo".

βAPN was shown to affect cell size and thus implies an affect on differentiation *in vitro* (Figure 3.5). Cells are larger and produce collagen X when they become hypertrophic (Wallis, 1996) and both of these observations are true when chondrocytes are cultured in the absence of βAPN (Figures 2.2 and 3.5) and therefore suggests that βAPN may induce dedifferentiation. There was no effect of βAPN on SLS aggregates as these were to be found regardless of the presence of βAPN and

they did not differ in length (data not shown). This indicates that cross-link formation in SLS assembly is not important.

3.4.2 ECM Morphology

The results presented here show the presence of SLS aggregates rather than fibrils in both the CM and the FRM (Figures 3.7 and 3.8). The chondrocytes in alginate bead culture were shown to remain phenotypically stable over a 14 day culture period and have previously been shown to remain stable for up to 8 months (Hauselmann *et al.*, 1994). The lack of fibrils present in the ECM produced by chondrocytes in alginate bead culture indicates that the collagen network is not crucial to phenotypic stability of the chondrocytes.

The high amount of collagens II and IX recovered from the medium (Table 2.1) can now be explained by the finding that SLS aggregates substitute collagen fibrils in this culture system. SLS aggregates are much smaller than fibrils and therefore do not contain as many collagen monomers. Also, the relatively small amounts of SLS aggregates in the CM and FRM means that a large amount of collagen II does not assemble and is therefore soluble in the medium. It could potentially form SLS aggregates in this compartment, but this seems unlikely as it has been found that SLS aggregates readily dissociate into monomers in the medium (Hulmes *et al.*, 1983). Collagen IX *in vivo* is covalently attached to the surface of collagen II fibrils (Figure 1.5) and if the SLS aggregates are actually composed of collagen II, there may not be suitable binding sites for the attachment of collagen IX molecules and thus its solubility in the medium. Also, the absence of collagen IX in the CM is probably due to the relative deficiency of SLS aggregates, while the higher abundance of SLS aggregates in the FRM provides a greater surface area to which the collagen IX molecules can attach which correlates to the higher amount of collagen IX recovered from this compartment (Table 2.1).

The lack of collagen fibrils in this culture system is in contrast to the results of Hauselmann *et al.* 1996, who reported that the matrix produced by human articular chondrocytes in alginate bead culture is similar to that of native cartilage. It should

however be noted that no banded collagen fibrils were visible in either the CM or the FRM. Moreover, SLS aggregates have also been observed in alginate bead culture of bovine chondrocytes (Dr B. Petit, personal communication). The question therefore arises to why the SLS aggregates form in alginate bead culture.

3.4.2.1 Why do SLS Aggregates Form?

It has been known for many years that SLS aggregates can be formed *in vitro* when certain negatively charged molecules, such as ATP or chondroitin sulphate, are added to a solution of collagen in dilute acid (Gross *et al.*, 1954; Perez-Tamayo, 1972). The overall pH of this culture system was found to be neutral but this does not account for the local pH within the bead which may be acidic. Therefore, by analogy to the *in vitro* conditions required for their formation, the negatively charged alginate may induce formation of SLS aggregates. The effect of pH on collagen morphology has been directly demonstrated in the chick cornea and shows that normal diameter fibrils are only laid down in a very narrow pH range (Bard *et al.*, 1993). SLS aggregates have also been shown to form when highly sulphated GAG's are present in the medium of limb bud cultures (Merker *et al.,* 1978). Sulphated GAG's have a large negative charge that changes the normal aggregation pattern of collagen possibly due to the strong electrostatic interactions between the highly negatively charged GAG chains and the polar groups on the collagen molecule. This may also be the case in the presence of alginate, which would account for the amorphous, black material surrounding each SLS aggregate (Figure 3.9) and may indicate a direct interaction between the polyanionic alginate and the collagen molecules. This interaction would interrupt the normal assembly pattern and induce formation of SLS aggregates. Other factors, for example proteoglycans, may also be required for SLS assembly, suggested by observations in a case of osteopetrosis fetalis where the generation of SLS aggregates was accompanied by an alteration in proteoglycan production (Bonucci *et al.*, 1975).

Alternatively, the negatively charged alginate may interact with the positively charged N-propeptide of the collagen II molecule (see chapter 4) and if this was the

case then the monomers could only assemble into structures that had the N-termini either on the outer surface or at either extremity. SLS aggregates match this latter criteria as the collagen molecules aggregate with their N- and C-termini in register (Gross *et al.*, 1954). Thus the N-propeptides, with their associated alginate would protrude from the N-terminus.

3.4.2.2 Which Collagen Type(s) Comprise the SLS Aggregates?

The identity of the collagen type(s) that form the SLS aggregates was not directly demonstrated as electron microscopy immunolabelling experiments were unsuccessful, but based on the results of other experiments presented in this thesis a number of possibilities occur (Figure 3.14). Collagen II alone could form the SLS aggregates (Figure 3.14a) although this is likely to form structures similar to those formed by collagen I and therefore would not account for the unusual banding patterns observed here. Alternatively, the SLS aggregates could comprise of collagens II and IX (Figure 3.14b) as solid phase immunoassays indicate the interaction of these collagen types at the monomer level (Figure 4.9). If the SLS aggregates were comprised of collagens II and IX the black artefacts that surround the SLS aggregates could be accounted for: the cationic NC4 domain of collagen IX on the surface of the SLS aggregates would interact with the polyanionic alginate. A further possibility that arises is that the SLS aggregates are comprised of collagens II, IX and XI like that of native heterotypic cartilage fibrils (Figure 3.14c). Another possibility is collagen X, as this collagen type has been shown to form SLS aggregates *in vitro* with banding patterns similar to those presented here, but the lengths described (130nm) do not correspond to that observed (section 3.4.2.3; Kielty *et al.*, 1984).

3.4.2.3 Length of SLS Aggregates

The length of the SLS aggregates formed here was compared to that of other studies. SLS aggregates formed from procollagen I in cultures of chicken embryo tendon cells

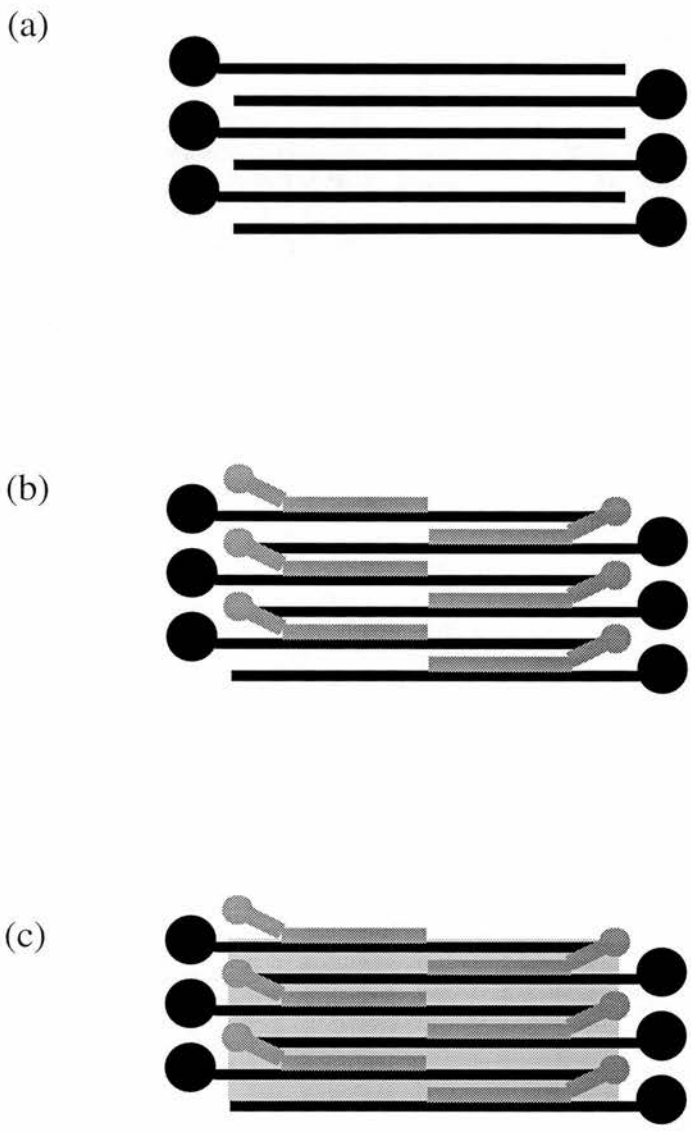


Figure 3.14. Models for the molecular composition of SLS aggregates in alginate bead culture. Models were made assuming that the composition of the aggregates was (a) collagen II alone, (b) collagen II and IX or (c) collagens II, IX and XI. ●—— represents collagen II, ⟍—— represents collagen IX and ▭ represents collagen XI.

were approx. 300 nm in length (Hulmes *et al.*, 1983) whereas the SLS aggregates formed here were on average 346nm long (Figure 3.10). These variances could be due to the SLS aggregates being composed of different collagen types or alternatively, differences in electron microscopy procedures and accuracy of measurements. SLS aggregates that were 346nm long were probably comprised mostly of collagen II, as their length is similar to the SLS aggregates formed from collagen I and it is the most abundant collagen recovered from both the CM and the FRM (Table 2.1). This correlates to the 346nm species being the most frequent length of SLS aggregate recovered.

SLS aggregates were also present with lengths in the range 100nm to 600nm (Figure 3.10). The shorter SLS (150nm) may be composed of collagen X as this collagen type has been found to form SLS aggregates of length 130nm (Kielty *et al.*, 1984). The small number of these shorter SLS aggregates correlates to the small amount of collagen X recovered from the CM (Figure 2.2). Alternatively, these shorter SLS aggregates may represent the 300nm species that had become cleaved or that had undergone partial proteolysis. In contrast, the longer SLS aggregates present (600nm) may depict aggregation of two of the 300nm species.

3.4.3 Final Conclusions

This chapter showed the phenotypic stability of chondrocytes in alginate bead culture over a 14 day culture period. The finding that SLS aggregates substituted collagen fibrils in both the CM and the FRM suggests that the collagen network is not crucial to phenotypic stability of the chondrocytes. It was shown by *in vitro* reconstitution experiments that alginate inhibited normal fibril assembly and thus it can be concluded that other suspension cultures or *in vitro* techniques must be employed to investigate cartilage morphogenesis.

CHAPTER 4

BIOCHEMICAL STUDIES ON CHICK CARTILAGE COLLAGENS

4.1 INTRODUCTION

Fibrillar collagens are secreted from cells in the form of procollagen, a larger precursor that contains extension propeptides at both the N- and C-termini of each α chain (Figure 4.1; Morikawa *et al.,* 1980). The conversion of procollagen to collagen requires the removal of both the propeptides by two different metalloproteinases, called procollagen N- and C-proteinase respectively (Tuderman and Prockop, 1982). N-proteinase cleaves a Pro-Gln bond in the pro-α1(I) chain and an Ala-Gln bond in the pro-α2(I) chain (Hojima *et al.,* 1994b) while C-proteinase/BMP-1 cleaves an -X-Asp- bond, where X marks the junction between the C-telopeptide and C-propeptide (Kadler and Watson, 1995). The sequential action of these 2 enzymes generates intermediates in the conversion of procollagen to collagen: the pN-collagen intermediate contains the N-propeptide but not the C-propeptide whereas the pC-collagen intermediate contains the C-propeptide but not the N-propeptide (Kielty *et al.*, 1993).

Procollagen N-proteinase is a neutral metalloproteinase that can be purified from a variety of tissues especially tendons and ligaments (Kadler *et al.,* 1995). The enzyme (260 kDa) was originally purified from calf tendon and shown to cleave the N-terminal peptide of procollagen *en bloc* (Kohn *et al.,* 1974). Later, an identical N-proteinase was recovered from chick embryo tendon, and was found to cleave the N-propeptide from procollagens I and II (Tuderman *et al.,* 1978) but not from procollagen III (Tuderman and Prockop, 1982). A new form of procollagen I N-proteinase (500 kDa) was identified and shown to consist of 4 subunits (161, 135, 120 and 61 kDa in chick tendons and 190, 175 125 and 58 kDa in bovine tendons; Hojima *et al.,* 1989; Hojima *et al.,* 1994b) and recently, another form of N-proteinase (107 kDa) was identified in bovine skin and was shown to have full enzymatic activity on a procollagen I substrate (Colige *et al.,* 1995).

The appearance of pN-collagen, due to a reduced activity of the N-proteinase enzyme, has been found in a number of diseases (Steinmann *et al.,* 1993). The heritable disorder dermatosparaxis, for example, is characterised by a markedly reduced activity of N-proteinase and therefore an accumulation of pN-collagen, and

leads to extreme fragility of the skin (Lapiere *et al.,* 1971). N-proteinase activity has also been linked to osteogenesis imperfecta and Ehlers-Danlos syndrome type VII, where mutations of procollagen genes alter the structure of the procollagen and cause either a decrease or a cessation of N-proteinase respectively (Kadler *et al.,* 1995). Kashin-Beck disease shows an accumulation of pN-collagen II in articular cartilage due to a structural alteration of procollagen II and thus the impaired conversion to collagen II by the N-proteinase (Yang *et al.*, 1991).

The answer to the question of what factor(s) control fibril diameter *in vivo* has long been sought after. Collagen fibrillogenesis assays in the presence of proteoglycans and other ECM components have often been used for these studies (Vogel *et al.*, 1984; Birk *et al.*, 1990; Kadler *et al.*, 1996) and recently a cell-free system of fibril formation has been developed where the N- and C-propeptides of procollagen are cleaved to collagen by the procollagen proteinases (Kadler *et al.*, 1990; Prockop and Hulmes, 1994). This system was introduced as an alternative to traditional *in vitro* reconstitution experiments, using fully processed collagen monomers, where assembly is initiated by neutralising and heating acidic solutions to physiological buffer conditions, using either "cold start" or "warm start" techniques (sections 3.2.5 and 4.2.8; Wood, 1960; Williams *et al.*, 1978; Holmes *et al.*, 1986). Whichever system is used to study fibril formation *in vitro,* a characteristic fibril growth-time curve can be obtained, either spectrophotometrically or by sedimentation experiments, and reveals a triphasic assembly process. First, there is a lag phase, due to the time taken for pre-fibrils to reach a size that can be either detected by light scattering or pelleted by centrifugation. This is followed by a period of rapid growth and finally, when fibril assembly is complete, by a plateau region (Williams *et al.*, 1978). *In vitro* fibrillogenesis experiments have been used to study fibril formation of all the fibrillar collagen types (Adachi and Hayashi, 1985; Thom and Morris, 1991; Fertala *et al.*, 1994; Kadler *et al.*, 1996; Mizuno *et al*, 1997) and persistence of the N- and/or C-propeptides has marked effects on fibril morphology (Hulmes *et al.*, 1989; Mould *et al.*, 1990).

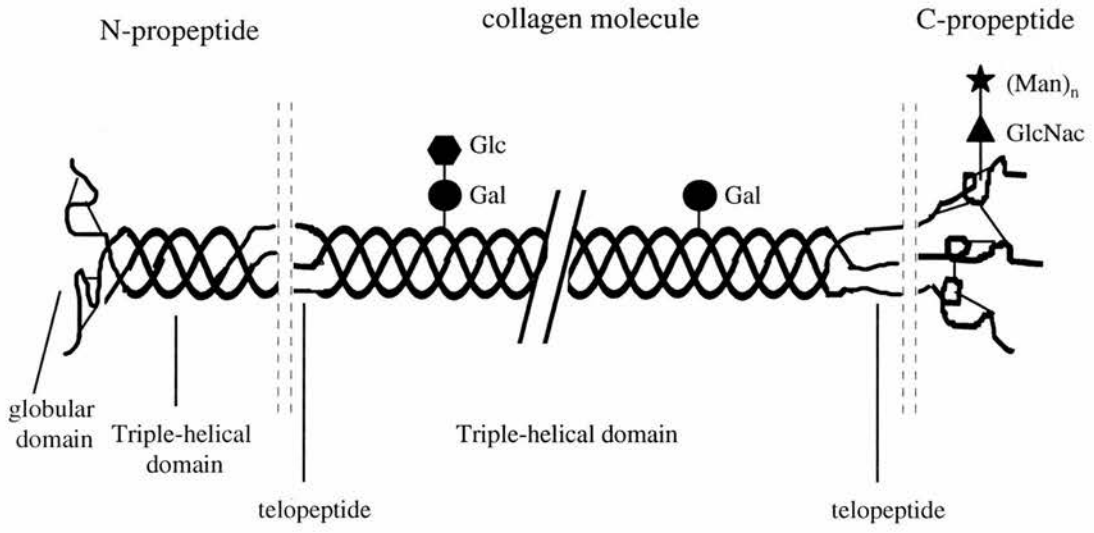


Figure 4.1. A procollagen type I molecule. Following secretion into the extracellular matrix the N- and C-propeptides are removed by N- and C- metalloproteinases respectively to leave the mature collagen molecule. Glc - glucose; Gal - galactose; Man - mannose; GlcNac - N-acetylglucosamine. (Adapted from Pesciotta *et al.*, 1982).

This chapter investigates two specific questions: first, what determines the extent of processing of procollagen II in alginate bead culture and second, what is the nature of the interaction of collagen IX with collagens II and XI *in vitro*?. It was found that large amounts of pN II (collagen II retaining the N-propeptide) accumulated in alginate culture. Procollagen N-proteinase did not cleave a purified pN II substrate, though this enzyme was active on pN II present in a crude mixture of proteins from alginate bead culture. The interactions of collagen IX with collagens II and XI were examined by ELISA assays and *in vitro* fibril formation experiments to investigate whether fibril formation is a pre-requisite for the binding of collagen IX molecules to collagen II, and, in view of the proposed surface location of the N-terminus of collagen XI on the heterotypic cartilage fibril, whether this collagen interacts with collagen XI during fibril assembly.

4.2 Materials And Methods

4.2.1 Materials

Insulin (bovine pancreatic), cysteine and polystyrene microtitre plates (Nunc-Immuno Plate IF, article no. 439454, AS) were from Gibco Life Technologies, Paisley, Strathclyde; Pyruvate was from Boehringer, Lewes, East Sussex; Glutamine, DEAE-Tris-acryl (plus M), dextran sulphate (sodium salt), $ZnSO_4.7H_2O$, Tween-20, bovine serum albumin (BSA), N-[tris(hydroxymethyl)methyl-2-amino]-ethanesulphonic acid (TES), hydrogen peroxide and o-phenyldiamine (OPD) were from the Sigma Chemical Co., Poole, Dorset; Horse radish peroxidase conjugated goat anti-rabbit IgG antibodies were obtained from the Scottish Antibody Production Unit, Carluke, Lanarkshire; N-proteinase (107 kDa) was a kind gift from Dr Alain Colige, Liege, Belgium; Procollagen I was a gift from Dr David Hulmes, Lyon, France; Collagen XIV was a kind gift from Dr Elisabeth Aubert-Foucher, Lyon, France; Crude mixtures of cartilage collagens from agarose cultures were kind gifts from Dr Jean Farjanel, Lyon, France and Mr Ulrich Blaschke, Münster, Germany; Heparin-

Sepharose, Concanavalin A and DEAE-Sephacel were from Pharmacia/LKB, Milton Keynes, Bucks; DE52 and CM52 were from Whatman, Maidstone, Kent. All other chemicals (analytical grade) were from BDH, Poole, Dorset, unless stated differently in the text.

4.2.2 Purification of Collagens II, IX and XI

4.2.2.1 Tris-acryl Chromatography

Partial purification of collagens from alginate bead culture were carried out as described previously (section 2.2.4). A Tris-acryl column (3cm x 60cm) was pre-equilibrated in start buffer (0.1M NaCl, 50mM Tris-HCl, 2M urea, pH 8.0) and the partially purified collagens, that had previously been dialysed against start buffer were loaded at a flow-rate of 40 ml/hr (Vaughan *et al.*, 1987). The column was washed with start buffer until the cpm reached background level and these breakthrough fractions (20ml each) were pooled and dialysed against heparin-Sepharose start buffer (section 4.2.2.2). Bound proteins were then eluted, stepwise, with 0.1M NaCl, 50mM Tris-HCl, 6M urea, pH 8.0, followed by this buffer containing increasing concentrations of NaCl (0.15, 0.2, 0.25 and 0.3M; Vaughan *et al.*, 1987). 100µl of each fraction was then added to 2.9ml of Ultima gold liquid scintillation cocktail (Packard) and the ^{3}H cpm determined using a Packard 1900 CA liquid scintillation analyser. Proteins in each peak were separated by SDS-PAGE and fluorography (6% separating and 4.5% stacking gels, under reduced and non-reduced conditions).

4.2.2.2 Heparin-Sepharose Chromatography

Heparin-Sepharose chromatography was used to purify collagen XI. Collagens XI and V specifically bind to heparin (Mizuno and Hayashi, 1994). A heparin-Sepharose column (4cm x 5cm) was pre-equilibrated in start buffer (0.1M NaCl, 10mM Tris-HCl, 2M urea, pH 7.5) and the breakthrough fractions from the Tris-acryl column (section 4.2.2.1) were loaded at a flow rate of 30 ml/hr. The column was then washed

with start buffer until the cpm reached background level while 20ml fractions were collected. Bound proteins were eluted with a linear gradient of 0.1M to 0.6M NaCl in start buffer and 100µl of each fraction was used for scintillation counting (see above). Proteins in each peak were analysed by SDS-PAGE and fluorography (6% separating and 4.5% stacking gels, under reduced and non-reduced conditions).

Fractions from both the Tris-acryl and the heparin-Sepharose columns, containing the collagens of interest, were dialysed against storage buffer (0.4M NaCl, 100mM Tris-HCl, pH 7.4) and stored at -70 °C until required.

4.2.3 Identification of the Biosynthetic Precursor

To identify the precursor form of collagen II found in alginate cultures, concanavalin A chromatography was carried out (Choglay *et al.*, 1993). A sample of partially purified collagens from alginate bead culture (section 2.2.4) was dialysed against start buffer (0.15M NaCl, 50mM Tris-HCl, 2M urea, 2mM $CaCl_2$, pH 7.5) while a concanavalin A column (1cm x 6cm) was pre-equilibrated in start buffer. The sample was loaded at a flow-rate of 20 ml/hr and 1ml fractions were collected. The column was washed with start buffer until cpm reached background levels and bound proteins were eluted with start buffer containing 0.5M α-methyl-D-mannoside. 100µl of each fraction was then added to 2.9ml of Ultima gold liquid scintillation cocktail (Packard) and the ^3H cpm determined using a Packard 1900 CA liquid scintillation analyser. The proteins in each fraction were then analysed by SDS-PAGE (6% stacking and 4.5% separating gels, under reducing conditions) and silver staining (A.2.2).

Further analysis of the biosynthetic precursor was also investigated. SDS-PAGE gels (6% separating and 4.5% stacking gels) of partially purified collagens were run under both non-reducing and reducing conditions, and also after pepsin digestion (section 2.2.5).

4.2.4 Serum-free Alginate Bead Culture of Chondrocytes

Chondrocytes were released from 17 day old chick sterna and suspended in alginate beads as described previously (section 2.2.2). Chondrocytes ($2x10^6$ cells/ml) were cultured for up to 1 month in DMEM containing 1% PS, 1% fungizone, 0.28mM L-ascorbic acid, 0.2mM βAPN, with either 10% FBS or in serum-free cultures. Serum-free cultures were carried out as described by Bruckner *et al.* 1989 where the medium contained the following supplements: 2mM glutamine, 100ng/ml insulin, 1mM cysteine and 1mM pyruvate (proline-free). Newly synthesised collagens were labelled from a stock medium containing 1μCi/ml [5-^{3}H]proline where the medium was changed every third day.

Collagens from the medium were partially purified as described previously (section 2.2.4) and analysed by SDS-PAGE with 6% separating and 4.5% stacking gels (reduced conditions), loading an equivalent volume in each lane. The gels were examined by fluorography and the relative molar percentage of each collagen chain was measured by densitometry (Chromoscan 3, Joyce Loebl).

4.2.5 Assay of Procollagen N-Proteinase

N-proteinase (107 kDa) was a kind gift from Dr Alain Colige, Liege, Belgium. The enzyme was supplied in 0.5M NaCl, 50mM Tris-HCl, 2mM $CaCl_2$, 0.02% Brij, pH 7.5 at a concentration of 30μg/ml. Enzyme activity was such that 1μl of N-proteinase completely processed 1μg of pN-collagen I in 16 hours at 37 °C. The following N-proteinase assay was based on that described by Colige *et al.*, 1995.

Substrate solution (30μl of procollagen (160μg/ml) or pN II (60μg/ml), in storage buffer) was mixed with 10μl of digest buffer (0.5M NaCl, 50mM Tris-HCl, 2mM $CaCl_2$, 0.02% Brij, 2.5mM NEM, 0.5mM PMSF, pH 7.5), in the presence or absence of N-proteinase. After incubation for 16 hours at 37 °C, the reaction was stopped by the addition of 20μl of 10mM EDTA, 50mM Tris-HCl, pH 7.5. Each sample was analysed by SDS-PAGE (6% separating and 4.5% stacking gels) and proteins visualised by silver staining (section A.2.2).

The crude mixture of proteins required for certain assays was prepared from alginate bead culture. Briefly, after 7 days of culture and solubilisation of the alginate, the CM (corresponding to approx. 2 x 10^6 cells) was collected by centrifugation (300g for 6 mins) and resuspended in 0.5ml PBS. This was then diluted 1:1 with assay buffer, in the presence or absence of N-proteinase. For investigations into a potential co-factor of N-proteinase, the above assay was repeated in the presence of 5µl each of collagens IX (50µg/ml in storage buffer) and XI (100µg/ml in storage buffer) or 10µl of collagen XIV (100µg/ml in 0.5M NaCl, 10mM Hepes, pH 7.4).

4.2.6 Purification of Collagen II for Interaction Studies

Due to the persistence of the N-propeptide on collagen II purified from alginate bead cultures, it was necessary to resort to another suspension culture system to produce collagen II for the *in vitro* interaction studies. An agarose culture system was chosen (Benya and Schaffer, 1982) in which 17 day old chick chondrocytes were cultured in agarose gels for up to 21 days, in the presence of 10% FBS (days 1 to 7) or 100ng/ml insulin (days 8 to 21). These cultures were carried out by Dr Jean Farjanel, Lyon, France, and Mr Ulrich Blaschke, Münster, Germany, who supplied a crude extract of collagens (in 1M NaCl, 0.1M Tris-HCl, 10mM EDTA, pH 7.4, 0.02% sodium azide) for subsequent purification.

The collagens were precipitated by the addition of solid NaCl until a final concentration of 4.2M was reached. This was stirred gently for 24 hours at 4 °C before collection of the collagens by centrifugation (20 000g for 45 mins at 4 °C). The pellet was resuspended in 1M NaCl, 100mM Tris-HCl, 10mM EDTA, pH 7.4 and dialysed against DE52 start buffer (0.1M NaCl, 100mM Tris-HCl, 2M urea, pH 7.4).

The sample was loaded onto a DE52 column (1.5cm x 16cm) that had been pre-equilibrated in start buffer, at a flow rate of 10ml/hr. The column was washed with 3 column volumes of start buffer and bound proteins were eluted with a linear gradient of 0.1M to 1M NaCl in start buffer. Fractions (5ml) were collected and their absorbances read at 220nm (Pharmacia Biotech Ultraspec 2000 UV/VIS

spectrophotometer). The breakthrough fractions were pooled and dialysed against CM52 start buffer (0.1M NaCl, 10mM Tris-HCl, 2M urea, pH 7.4). Fractions that contained collagen IX were dialysed against storage buffer and stored at -70 °C until required.

The dialysed sample was loaded onto a CM52 column (1.5cm x 16cm) that had been previously equilibrated in CM52 start buffer, at a flow rate of 40ml/hr. The column was then washed with 3 column volumes of CM52 start buffer and bound proteins were eluted stepwise with 0.5M NaCl in start buffer. Fractions (5ml) were collected and their absorbances read at 220nm. The breakthrough fractions were pooled and dialysed against low salt DE52 start buffer (5mM NaCl, 50mM Tris-HCl, 2M urea, pH 7.4). Fractions that contained collagen XI were further purified on a heparin-Sepharose column (section 4.2.2.2) before dialysis against storage buffer and storage at -70 °C.

A second DE52 column (1.5cm x 16cm) was pre-equilibrated in low salt DE52 start buffer and the sample was loaded at a flow rate of 40ml/hr. After extensive washing of the column with start buffer, bound proteins were eluted stepwise, with 0.5M NaCl in start buffer. Fractions were collected (5ml) and the breakthrough fractions were pooled and dialysed against heparin-Sepharose start buffer (0.1M NaCl, 10mM Tris-HCl, 2M urea, pH 7.5). The heparin-Sepharose column was run as described previously (section 4.2.2.2) and fractions that contained purified collagens II and XI were dialysed against storage buffer and stored at -70 °C until required.

4.2.7 Solid Phase Assay

A modification of the ELISA technique was used to measure the binding of collagen IX to collagen II monomers immobilised on the wells of a microtitre plate (Hedbom and Heinegard, 1989). Collagen II was purified from agarose culture while collagen IX was purified from alginate bead culture. As collagen IX was purified by ion exchange chromatography (Figure 4.2), the proteoglycan form of the molecule was used in these experiments.

Collagen II monomers were prepared by diluting a stock solution of collagen (300µg/ml) with storage buffer (0.4M NaCl, 100mM Tris-HCl, pH 7.4) to obtain a final concentration of 1µg/ml. 200µl of this collagen solution was then added to the wells of a polystyrene microtitre plate (Nunc-Immuno Plate IF, article no. 439454, AS Nunc), and incubated for 16 hours at 4 °C in a humidity chamber. The absorbed protein was cross-linked by addition of 0.25% (v/v) glutaraldehyde in 0.15M NaCl, 0.5mM $NaCNBH_4$, 20mM TES, pH 7.4 for 2.5 hours at 37 °C, and any remaining reactive groups were then blocked by addition of 0.2M ethanolamine in the same buffer for 30 mins at 37 °C. After extensive washing (5x200µl) with PBS2 (0.14M NaCl, 5mM sodium phosphate, 0.05% Tween-20, pH 7.4), non-specific binding was blocked by the addition of PBS3 (PBS2 containing 100 µg/ml BSA) for 4 hours at room temperature with agitation. Wells were washed with PBS4 (5x200µl; 0.14M NaCl, 30mM sodium phosphate, 0.05% Tween-20, pH 7.4) before adding 200µl/well of various dilutions of collagen IX in PBS4 (see Figure 4.9 for details). The collagens were incubated overnight at room temperature with agitation for binding to occur, before washing with PBS4 (5x200µl). Primary antibody (200µl/well of affinity purified anti-collagen IX antibody at 1:100 dilution) was added to each well and incubated overnight at 4 °C. After a further washing step (5x200µl of PBS4), 200µl/well of secondary antibody (horse radish peroxidase (HRP) conjugated goat anti-rabbit antibody, 1:5000 dilution) was incubated for 2 hours at room temperature. Following the final wash (5x200µl PBS4), 200µl of o-phenyldiamine dihydrochloride (0.4mg/ml in phosphate-citrate buffer (50mM Na_2HPO_4, 24mM citric acid, pH 5.0) containing 0.4µl/ml H_2O_2) was added to each well and incubated in the dark for 40 mins. The reaction was stopped by the addition of 50µl/well of 3M sulphuric acid and the absorbance of each well was measured immediately in a Dynatech MR7000 microplate reader. All experiments were carried out in triplicate with appropriate controls where collagen II and/or collagen IX were replaced by BSA and PBS4 respectively.

4.2.8 Fibril Formation Assay

To study the interaction of collagens IX and XI, collagen XI fibrils were formed using the "warm start" technique, where both the collagen and mixing solutions are pre-warmed separately prior to fibril assembly (Holmes *et al.*, 1986). Collagen XI fibrils were also formed in the presence of collagen IX.

Collagen XI (500µg/ml) in storage buffer (0.4M NaCl, 100mM Tris-HCl, pH 7.4) and dH_2O containing 100µg/ml BSA were preincubated separately at 37 °C in 0.5ml eppendorf tubes. The inclusion of BSA in this assay prevents non-specific interactions of the protein of interest (collagen XI) with the walls of tubes and with pipette tips but does not interfere with the assembly process (Brown and Vogel, 1989). The collagen solution (10µl) and dH_2O were mixed 1:1 to initiate fibril formation and at the times indicated (Figures 4.10 and 4.11), the fibrils were pelleted by centrifugation (9 500g for 10 mins). The supernatant and pellet (resuspended in an equal volume of storage buffer) were collected and analysed by SDS-PAGE (6% separating gels and 4.5% stacking gels) and Coomassie staining. Identical assays were performed where the amount of protein in the pellet and supernatant was estimated by the BCA assay (section A.5).

The interactions of collagens IX (^{3}H-labelled) and XI (nonradio-labelled) were investigated by *in vitro* reconstitution experiments. Collagen XI fibrils were formed as described above in the presence of collagen IX (200µg/ml). Following centrifugation (9 500g for 10 mins), the supernatant and pellet (resuspended in an equal volume of storage buffer) were collected. As collagen IX was the only radioactive component of this fibril formation assay, its location either in the supernatant or the pellet could be directly assessed. The supernatant or the resuspended pellet was added to 3ml of Ultima gold liquid scintillation cocktail (Packard) and ^{3}H cpm were determined using a Packard 1900 CA liquid scintillation analyser. Collagen IX/XI fibrils were also assessed by SDS-PAGE and western blotting (6% separating gels and 4.5% stacking gels under reduced conditions). Affinity purified antibodies to collagen IX were diluted 1:3000, while horse-radish peroxidase (HRP) conjugated goat anti-rabbit secondary antibodies were diluted

1:2000 in TBST (0.15M NaCl, 50mM Tris-HCl, 0.05% (v/v) Tween-20, pH 7.9). Immunoreactive proteins were then detected by ECL (section A.3.2).

4.3 RESULTS

As this chapter investigates 2 specific questions, this results section is subdivided into parts. The first section (4.3.1) identifies the precursor form of collagen II as pN II and examines methods for removal of the pN II from collagen II preparations. Investigations included omitting FBS from the culture medium and using procollagen N-proteinase to cleave the N-propeptide from pN II. The second section (4.3.2) describes the use of ELISA and *in vitro* fibrillogenesis techniques to investigate the interactions between collagen IX and collagens II and XI at both monomer and fibril levels.

4.3.1 Purification of Collagens II, IX and XI

Collagens II, IX and XI from alginate bead culture were purified by DEAE Tris-acryl and heparin-Sepharose chromatography (Figures 4.2 and 4.3). Collagen IX eluted from the Tris-acryl column between 0.2M and 0.25M NaCl and therefore represents the proteoglycan form of collagen IX (Figure 4.2a). The large peak at the 6M urea elution step (Figure 4.2a) represents other proline-containing proteins for example the non-proteoglycan form of collagen IX (section 2.3.1.3), as well as collagen X. Collagen II eluted from the heparin-Sepharose column at approx. 0.18M NaCl while some collagen XI eluted at approx. 0.28M and additional collagen XI eluted at 0.33M NaCl (Figures 4.3a and b). There are 5 bands that represent collagen XI and the top two probably represent either variants of the α1(XI) chain due to alternative splicing of the PARP domain (section 1.3.3.2), or partially processed precursors of collagen XI.

Following the above purification, collagen II was found to be present as 2 bands, that represents up to 50% of the collagen II recovered (Figures 4.4a). These were assumed to represent collagen II and a precursor form of collagen II as following pepsin

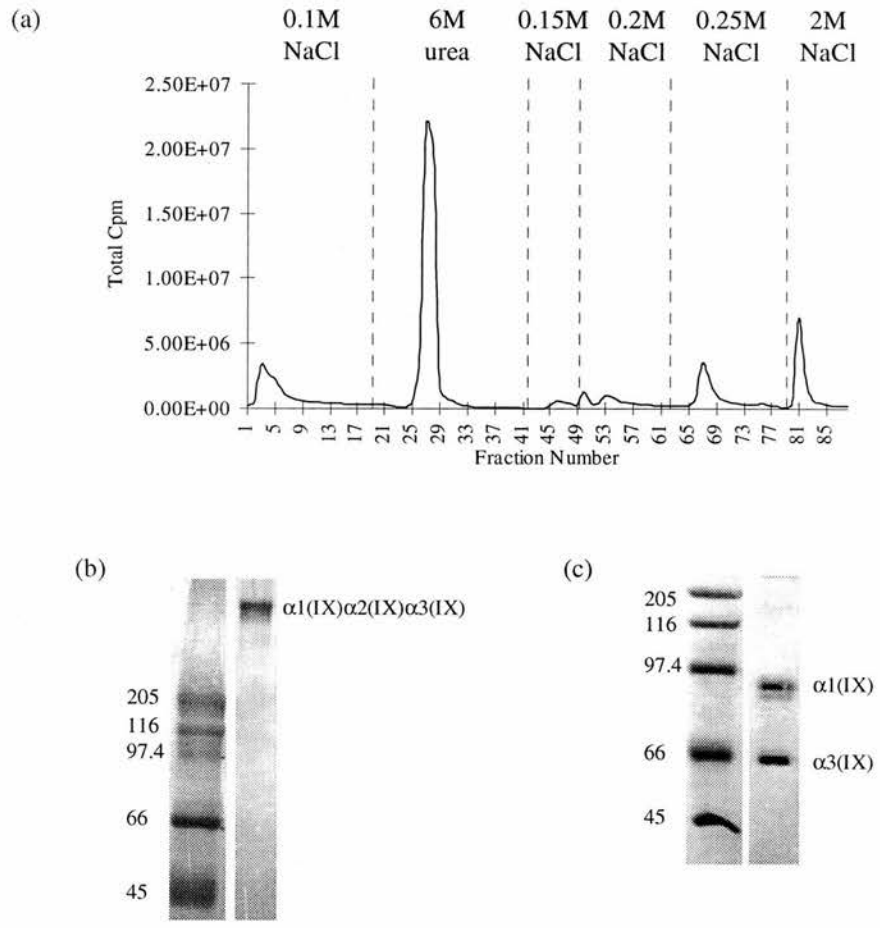


Figure 4.2. Separation of partially purified collagens by Tris-acryl chromatography. Partially purified collagens were dialysed against Tris-acryl start buffer (0.1M NaCl, 50mM Tris-HCl, 2M urea, pH 8.0) and the sample was loaded onto the column at a flow rate of 40 ml/hr. Breakthrough fractions containing collagens II and XI were collected for heparin-Sepharose chromatography. After washing, bound proteins were eluted stepwise with start buffer containing 6M urea and 0.1M, 0.15M, 0.2M, 0.25M and 2M NaCl in as indicated (a). Collagen IX eluted between 0.2M and 0.25M NaCl. (b) 6% non-reduced fluorogram of collagen IX with molecular weight markers (kDa). (c) 6% reduced fluorogram of collagen IX with molecular weight markers (kDa).

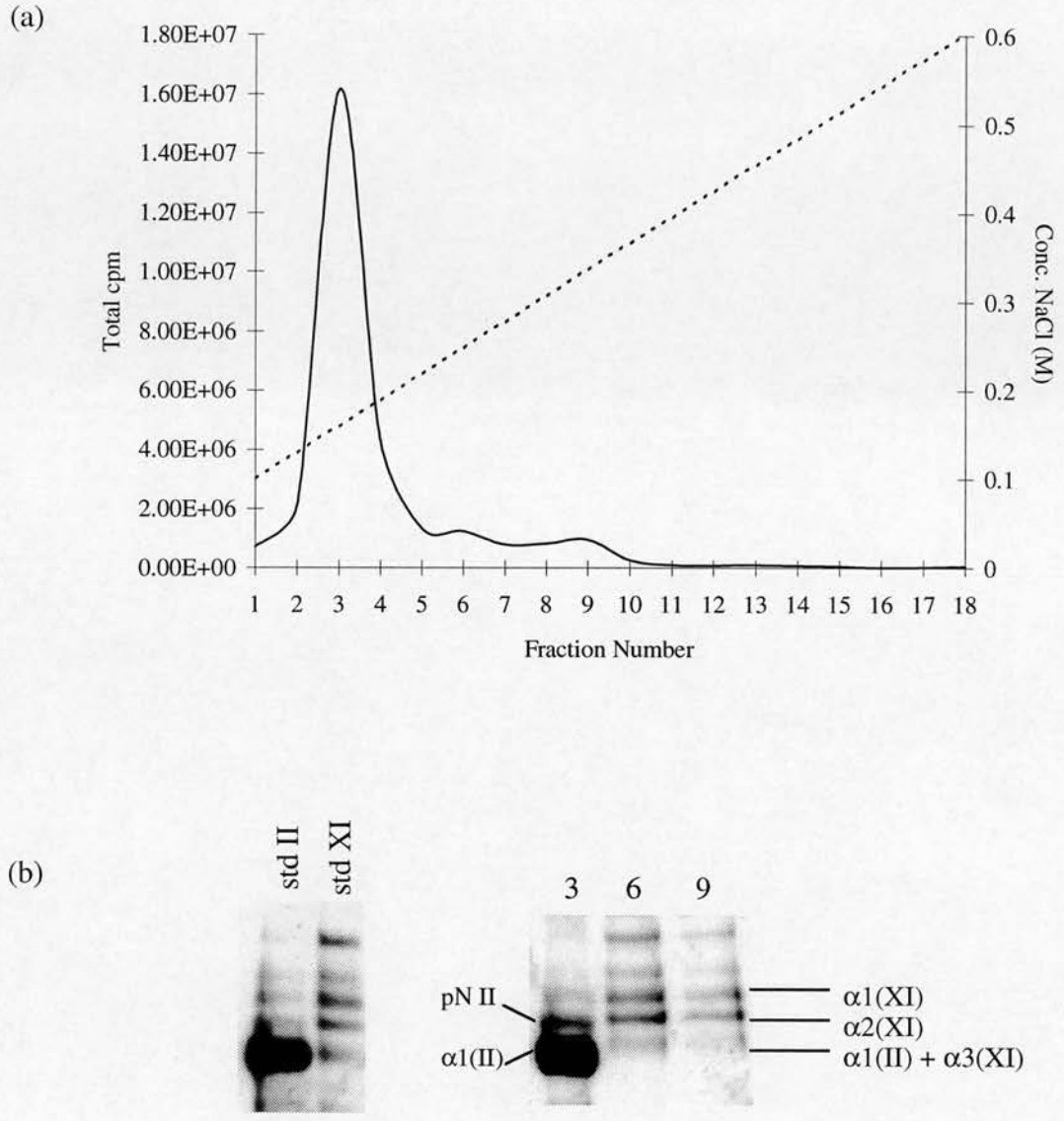


Figure 4.3. Heparin-Sepharose chromatographic separation of collagens II and XI. (a) The breakthrough fraction from the Tris-acryl column was dialysed against heparin-Sepharose start buffer (0.1M NaCl, 10mM Tris-HCl, 2M urea, pH 7.5) and loaded onto the column at a flow rate of 30 ml/hr. After washing, bound proteins were eluted with a linear gradient from 0.1 to 0.6M NaCl in start buffer. Collagen II (including pN II) eluted at approx. 0.18M NaCl and collagen XI between 0.28M and 0.33M NaCl. (b) the fluorogram (6% separating gel) shows that some collagen XI eluted in the first peak while additional collagen XI eluted with a higher concentration of NaCl.

digestion, one band was present and showed increased intensity on the fluorogram (Figure 4.4a). Furthermore, SDS-PAGE reduced and non-reduced data indicate that the precursor is pN-collagen II since in procollagen type II, interchain disulphides are only found in the C-propeptide. This was further confirmed by the presence of 2 bands following concanavalin A chromatography which would bind sugar residues found in the C-propeptide (Figure 4.4b and c). Now that the identification of pN II as the biosynthetic precursor was established, methods of its removal were investigated.

4.3.2 Investigations into the Removal of pN II Collagen

4.3.2.1 Serum-free Alginate Bead Culture of Chondrocytes

As described above, (section 4.3.1), large amounts of pN II were found in the non-bound fraction from the DEAE Tris-acryl column, which eluted with collagen II on subsequent heparin-Sepharose chromatography (Figure 4.3). FBS contains many proteinase inhibitors that may prevent N-proteinase activity and thus explain the high amount of pN II observed. Therefore, 10% FBS was replaced by 100ng/ml insulin in the culture medium (Bohme *et al.*, 1992). It was found that the amount of pN II was reduced to 10% but analysis of total cpm suggested that total collagen production was also reduced (Figure 4.5). This result confirmed that proteinase inhibitors present in the serum prevented N-proteinase activity. Preliminary investigations indicated that the presence of 50μM $ZnSO_4$ to the medium of serum-free cultures also acted to decrease the relative amount of pN II (results not shown). However, as collagen II was required completely free from biosynthetic intermediates for *in vitro* interaction studies, other methods of reducing the amounts of pN II were examined.

4.3.2.2 N-proteinase Cleavage of the N-propeptide from pN II

The 107 kDa form of N-proteinase and collagen XIV were kind donations from Dr Alain Colige, Liege, Belgium and Dr Elisabeth Aubert-Foucher, Lyon, France respectively. The results of the following assays are summarised in Table 4.1.

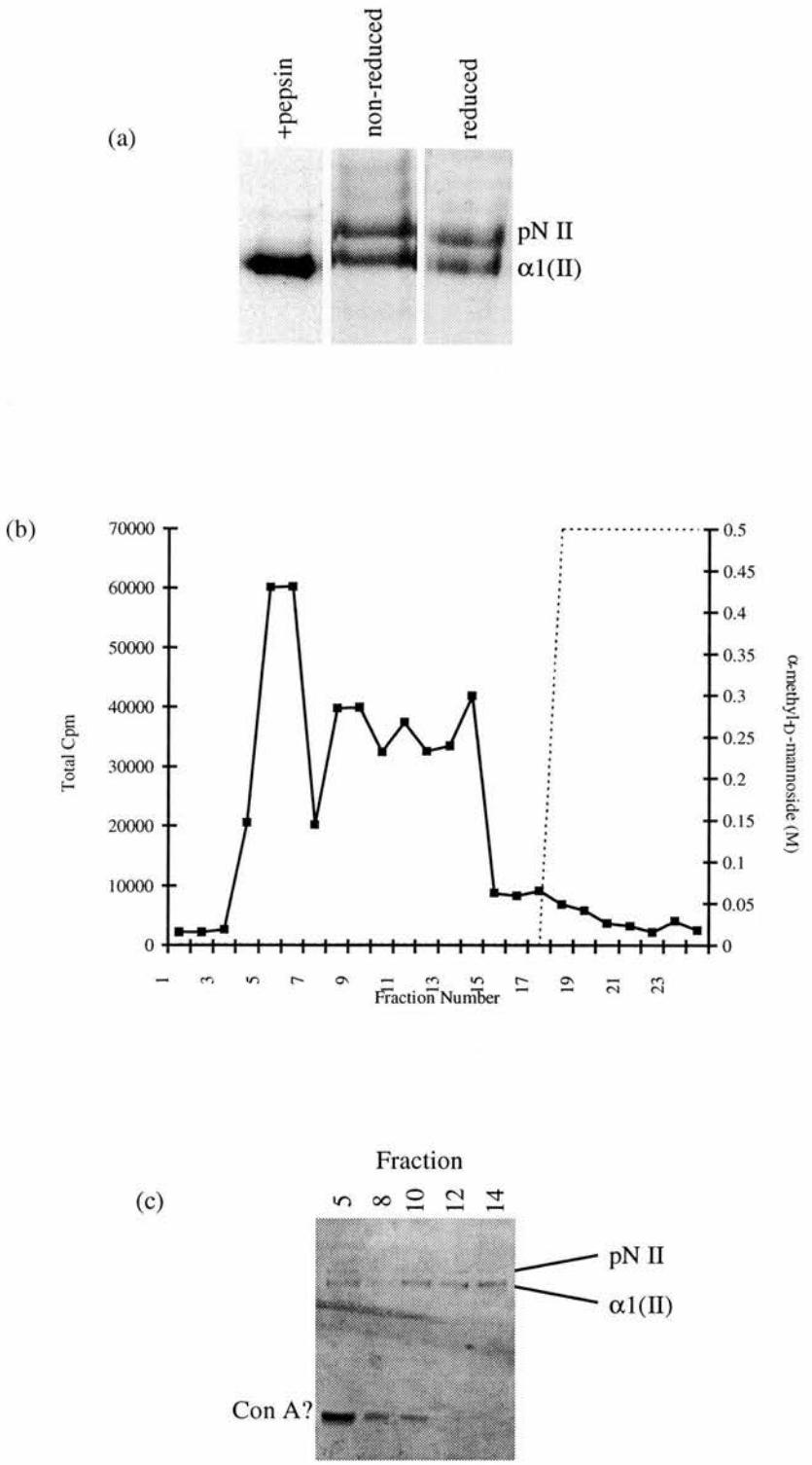
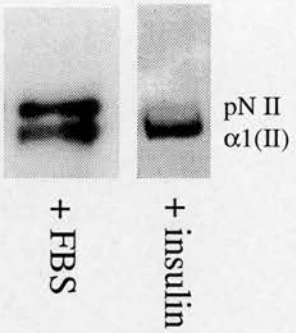


Figure 4.4. Identification of the biosynthetic precursor. (a) fluorograms showing the effect of reduction and pepsin digestion on partially purified collagens. (b) A concanavalin A column was run of partially purified collagens. The column was pre-equilibrated in start buffer (0.15M NaCl, 50mM Tris-HCl, 2M urea, 2mM $CaCl_2$, pH 7.5) and the sample loaded on at a flow-rate of 20 ml/hr. Bound proteins were eluted with 0.5M α-methyl-D-mannoside in start buffer. (c) SDS-PAGE analysis of fractions 5,8,10,12 and 14 that had been silver stained.



	pN II	collagen II
10% FCS	54%	46%
100ng/ml Insulin	10%	90%

	Total Cpm After Partial Purification
10% FCS	**900 000**
100ng/ml Insulin	**666 666**

Figure 4.5. Effect of replacing 10% FBS with 100ng/ml insulin in alginate bead culture. After partial purification of the collagens from each culture condition, it was found that the omission of FBS reduced the ratio of pN II:collagen II. Ratios of each band were quantified by densitometry. Note: the FBS lane has been examined by fluorography while the insulin lane has been stained with Coomassie blue. Total cpm indicated that collagen production was decreased by 30% when FBS was omitted from the medium.

Following incubation with the N-proteinase for 16 hours at 37 °C, the N-proteinase was found to be active on a procollagen I substrate, as expected (Figure 4.6a). This cleavage occurred even at low concentrations of enzyme. The pN II, purified by Tris-acryl and heparin-Sepharose chromatography (Figure 4.2 and 4.3), was also incubated with N-proteinase but it was found that there was no cleavage of the N-propeptide (Figure 4.6b). Increasing the N-proteinase concentrations showed that when the substrate and enzyme were in a 1:1 dilution there was a slight reduction in the amount of pN II but significant amounts remained (Figure 4.6c). When, however a crude mixture of proteins that included pN II was incubated overnight with the N-proteinase enzyme it was found that there was complete processing of the pN II, even at low enzyme concentrations (Figure 4.6d).

The observation that N-proteinase activity on pN II was only observed when this substrate was present in a crude mixture of proteins suggested that a possible co-factor was also required for activity. Preliminary investigations into the identity of this co-factor were performed. As the crude mixture of proteins from alginate bead culture also contained collagens IX and XI, it was hypothesised that the interaction of pN II with these other cartilage-specific collagens might be required for activity. Purified collagens IX and XI were therefore added to the assay on purified pN II/collagen II. It was found that N-proteinase remained inactive on a pN II substrate in the presence of these collagen types (Figure 4.7a).

The N-proteinase used in this study (107kDa) co-purifies and specifically binds to collagen XIV (Colige *et al.*, 1995). Therefore it was hypothesised that this FACIT collagen may guide docking of the N-proteinase to a specific cleavage site on pN II. Addition of collagen XIV to purified pN II in this assay system, however had no affect on the activity of N-proteinase (Figure 4.7b).

A number of other experiments were performed which took into consideration some of the factors that are required for N-proteinase activity *in vitro* (Figure 4.7). For example, zinc ions are crucial for the active site of the N-proteinase enzyme (Tanzawa *et al.*, 1985) and as preliminary experiments found that addition of $ZnSO_4$ to the alginate bead culture medium decreased the amount of pN II present (section

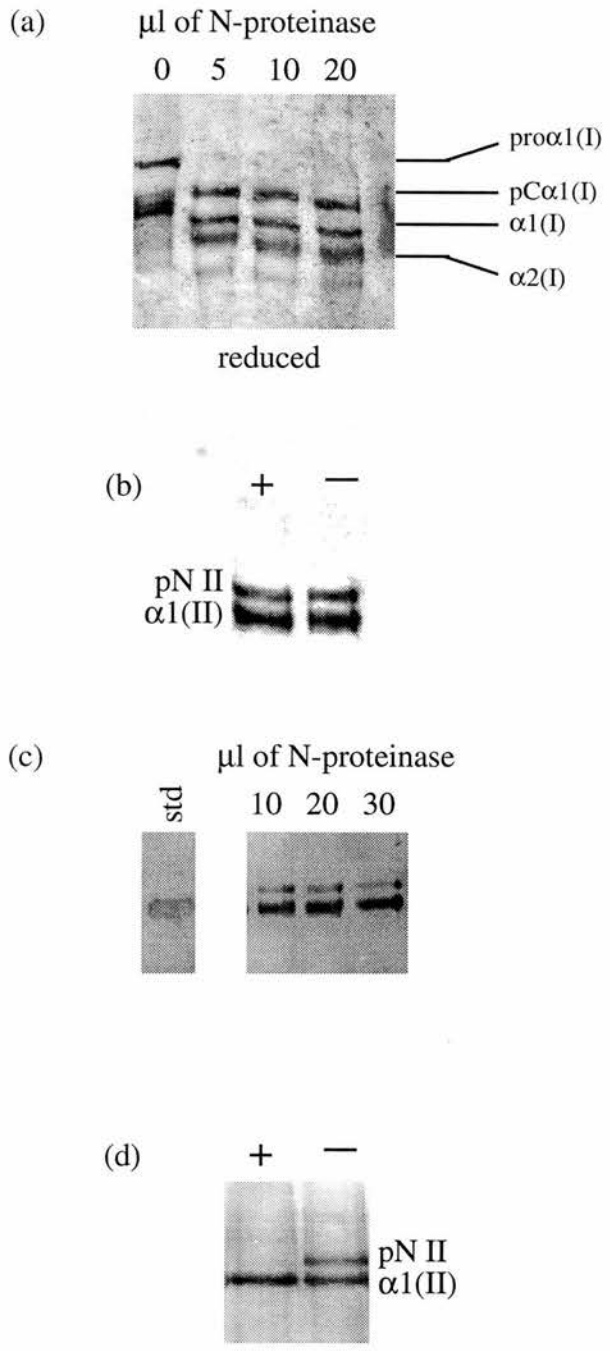


Figure 4.6. Effect of procollagen N-proteinase treatment. 30µl of collagen substrate was mixed with 10µl (or the volumes indicated) of assay buffer (-), or assay buffer containing N-proteinase (+). After incubation for 16 hours at 37 °C, the samples were analysed by SDS-PAGE (6% separating and 4.5% stacking gels) and silver staining. N-proteinase cleaves the N-propeptide from procollagen I at low enzyme concentrations (a). The enzyme is not active on purified pN II collagen (b) but there is a slight decrease in pN II when the substrate and enzyme (30µl) are in a mixed 1:1 ratio (c). N-proteinase cleaves the N-propeptide from pN II present in a crude mixture of proteins (d).

4.3.2.1), zinc was also added to this assay. Experiments were performed in the presence of both 15 and 50μM $ZnSO_4$ but there was still no cleavage of the N-propeptide from pN II (Figure 4.7d and e). Also, it has been found previously that N-proteinase activity can be increased if the procollagen is aggregated in the presence of dextran sulphate or polyethyleneglycol (Hojima *et al.*, 1994a) but the addition of 0.05% dextran sulphate to the assay system did not induce activity of the N-proteinase (Figure 4.7c).

4.3.2.3 Conclusions Concerning the Removal of pN II

The above assays show that pN II is extremely difficult to remove as there was only one method that was successful in its removal, that is, addition of N-proteinase to pN II present in a crude mixture of proteins (Figure 4.6d). It therefore seems likely that a co-factor is required for the activity of the N-proteinase enzyme on a collagen II substrate but preliminary investigations into its identity were unsuccessful. The results of all the N-proteinase assays are summarised in Table 4.1. Serum-free cultures are encouraging in that 90% of pN II is removed but these have the disadvantage that total collagen production is reduced by 30% (Figure 4.5).

4.3.3 *In Vitro* Interaction Studies of Cartilage Collagens

This section investigates the interactions of cartilage collagens at both monomer and fibrillar levels. The main questions addressed are: (1) do collagens II and IX interact as monomers (2) are collagen II fibrils a prerequisite for the attachment of collagen IX ? and (3) do collagens IX and XI interact during any stage of fibril assembly? These questions are examined using the ELISA technique and *in vitro* fibril formation experiments.

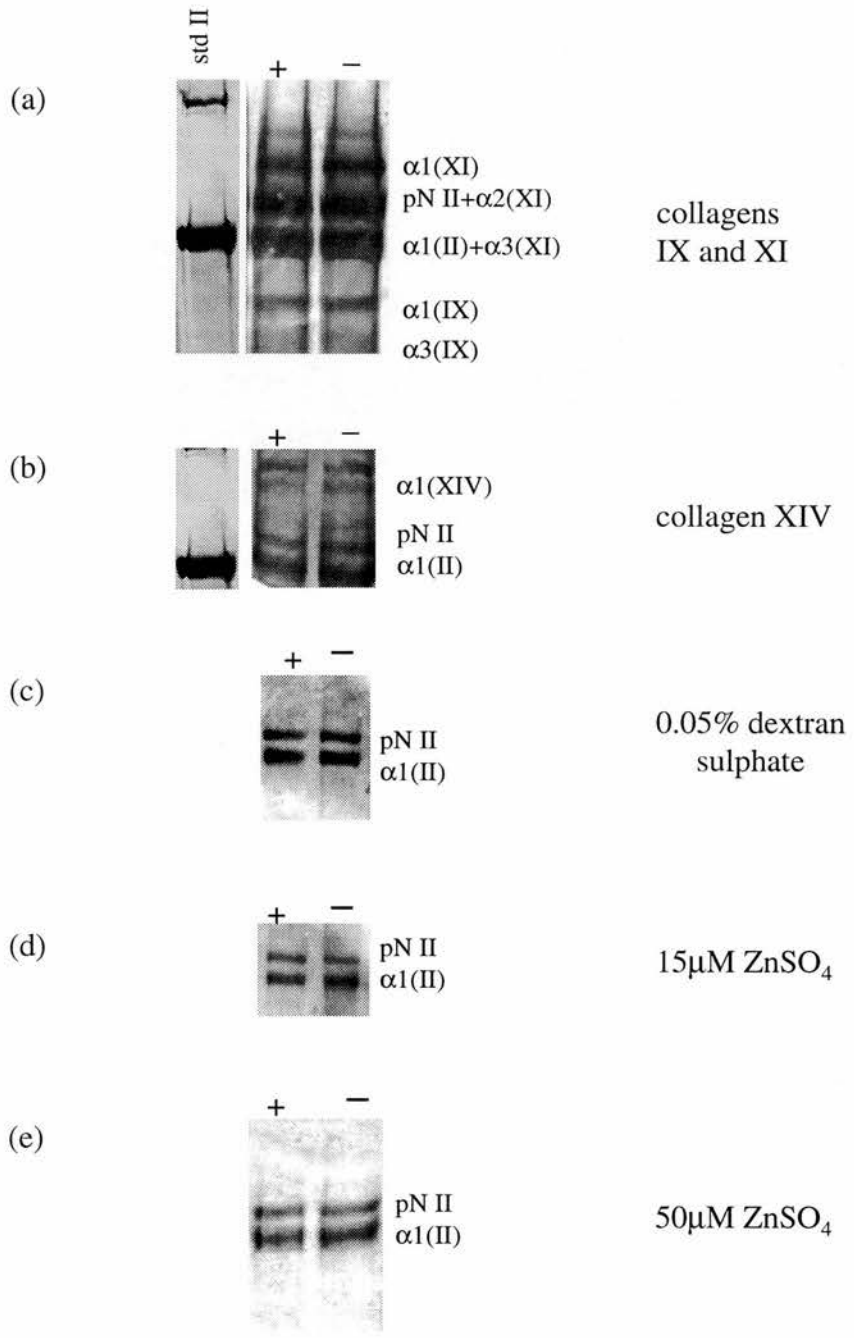


Figure 4.7. Further investigations into N-proteinase activity. The N-proteinase assay was carried out as described in Figure 4.6 in the presence of the following: (a) collagens IX and XI (50μg/ml and 100μg/ml respectively), (b) collagen XIV (100μg/ml), (c) 0.05% dextran sulphate, (d) 15μM $ZnSO_4$ and (e) 50μM $ZnSO_4$. Samples were analysed by SDS-PAGE (6% separating and 4.5% stacking gels under reduced conditions) and silver staining.

SUBSTRATES	PROCESSING?
purified pN II	X
pN II in crude extract	✓
procollagen I	✓
pN II + collagens IX and XI	X
pN II + collagen XIV	X
pN II + 0.05% dextran sulphate	X
pN II + 15μM ZnSO4	X
pN II + 50μM ZnSO4	X

Table 4.1. Summary of N-proteinase activity. ✓ indicates processing of the N-propeptide from pN II while **x** indicates that the enzyme was not active.

4.3.3.1 Purification of Collagen II from Agarose Cultures

The problems in obtaining pure, fully processed collagen II from alginate bead culture (section 4.3.2) meant that it was necessary to purify this collagen type from another suspension culture. Crude extracts of collagens from agarose gels were kind gifts from Dr Jean Farjanel, Lyon, France and Mr Ulrich Blaschke, Münster, Germany.

The crude extract, which contained collagens II, IX and XI, was purified by DE52, CM52, DE52 (under low salt conditions) and heparin-Sepharose chromatography. Elution profiles and SDS-PAGE analysis of the purified collagens at each column step are shown (Figure 4.8). Collagen XI was further purified on a heparin-Sepharose column to remove the residual collagen IX visible by SDS-PAGE. Recoveries at each step are summarised in Table 4.2.

For the following interaction studies, collagens II and XI were purified from agarose culture as above. Collagen IX (proteoglycan form) was purified from alginate bead culture as this collagen type was readily available in large quantities from the medium. It should also be noted that collagen IX was the only radioactive collagen present.

4.3.3.2 Interaction of Collagen II Monomers with Collagen IX

To study the interactions of collagens II and IX at the monomer level, the ELISA technique was used. Collagen II monomers (1μg/ml) were immobilised on the wells of microtitre plates before addition of collagen IX molecules at the following concentrations: 0.078, 0.156, 0.312, 0.625, 1.25, 2.5, 5 and 10μg/ml. The absorbance of each well was read and the absorbance values plotted as the mean of three values (Figure 4.9). It was found that as the concentration of collagen IX molecules increased there was a concomitant rise in absorbance which indicates that collagens II and IX interact at monomer level and collagen II fibrils are not a prerequisite for the attachment of collagen IX molecules.

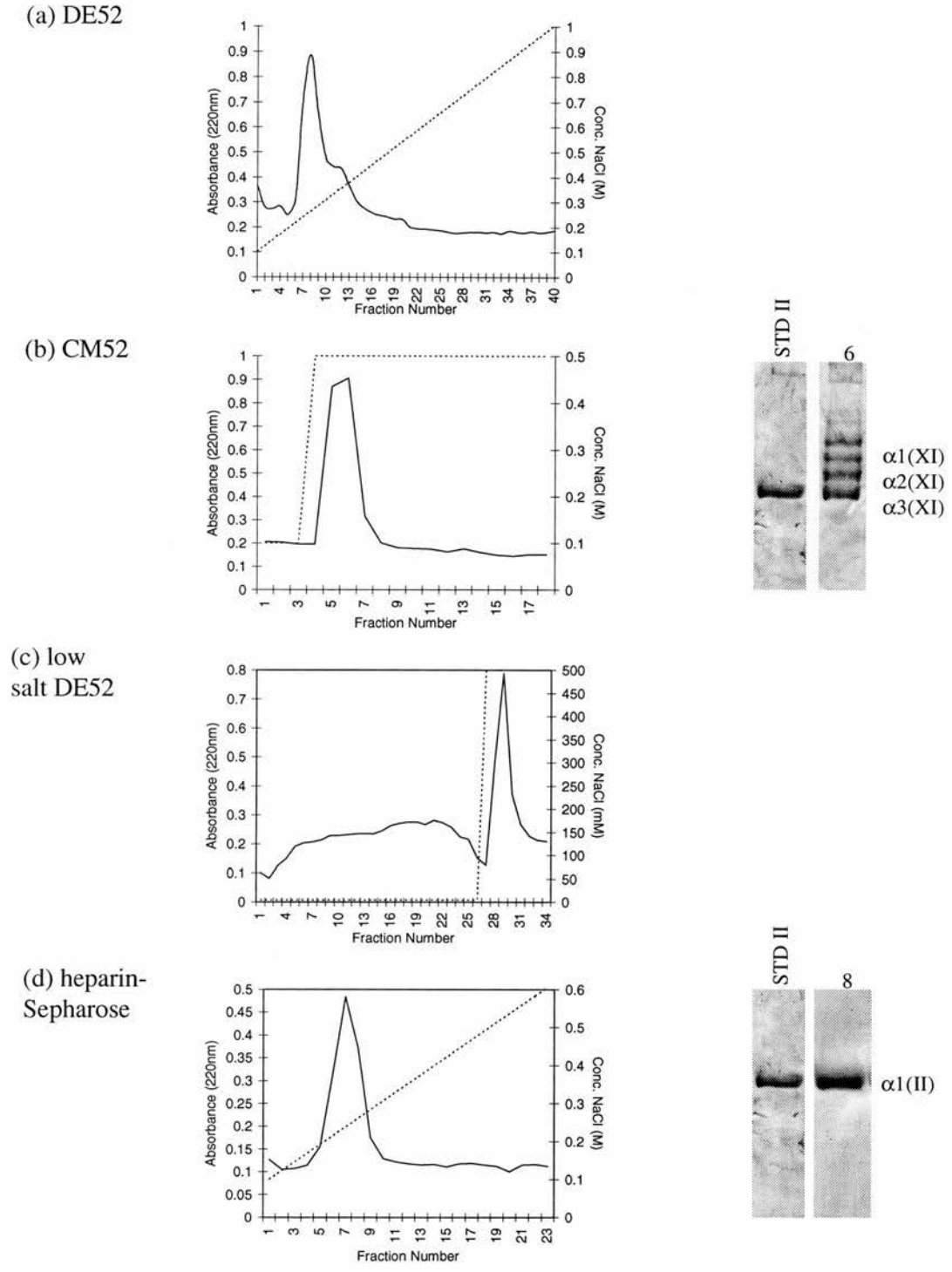


Figure 4.8. Elution profiles of the chromatographic steps involved in purification of collagen II from agarose culture. (a) DE52 column. (b) CM52 column. (c) low salt DE52 column. (d) heparin-Sepharose column. Gel lanes show the purified collagen XI (b) and collagen II (d) that were used in fibrillogenesis studies. The two bands that are unlabelled in the purified collagen XI lane probably represent variants of the α1(XI) chain due to alternative splicing in the N-terminal region (section 1.3.3.2).

STEP	AMOUNT OF PROTEIN (μg/ml)	VOLUME	TOTAL PROTEIN (μg)	% RECOVERY
Crude Extract	112	40	4480	-
4.2M NaCl Precipitate	181	22	3982	90
Loaded onto DE52	215	23	4945	100
Loaded onto CM52	52	80	4160	93
Loaded onto Low Salt DE52	35	105	3675	82
Loaded onto Heparin-Sepharose	25	96	2400	54
Following Concentration and Dialysis into Storage Buffer	1080	1.5	1620	36

Table 4.2. Recoveries of proteins at each chromatography step in the purification of collagen II.

4.3.3.3 Interactions of Collagens IX and XI

By analogy to collagen V, the N-terminus of collagen XI is thought to reside on the surface of the heterotypic cartilage collagen fibril (section 1.3.3.2) and this location would allow collagen XI to interact with collagen IX molecules. Collagen XI forms fibrils *in vitro* (Smith *et al.*, 1987) and therefore the interactions of collagens IX and XI were investigated by *in vitro* reconstitution experiments.

Collagen XI (500μg/ml) was reconstituted into fibrils by use of the "warm start" technique. Fibrils at each time point (0, 0.25, 0.5, 0.75, 1 and 4 hours) were collected by centrifugation and collagen XI in both the pellet and the supernatant were assessed by the BCA assay or were analysed by SDS-PAGE (Figure 4.10). Collagen XI forms fibrils with a classical triphasic kinetic assembly curve, with most of the monomers assembled into fibrils by 1 hour (Figure 4.10a). At time point zero, however, there was a higher percentage of collagen XI in the pellet than at the 0.25h time point. SDS-PAGE analysis confirmed that most of the collagen XI was present in the pellet by 1 hour but did not confirm that collagen XI appeared as a higher percentage in the pellet at time point zero (4.10b). This anomaly may be due to the presence of small molecular weight proteins which would not be detected on a 6% SDS-PAGE gel.

Collagen XI (500μg/ml) fibrils were also reconstituted in the presence of collagen IX (200μg/ml; Figure 4.11). As collagen IX was the only radioactive component of the fibril formation assay, its location (either in the pellet or the supernatant) could be directly assessed by scintillation analysis (Figure 4.11a). It was shown that the amount of collagen IX present in the pellet exactly mirrored the fibril formation curve of collagen XI, including the increased amount found in the pellet at time point zero (Figure 4.11a). Western blotting with polyclonal antibodies to collagen IX showed that this collagen type was present in the pellet where it appeared in a higher amount at time point zero than at the 0.25h time point (Figure 4.11b). In both the cpm and western blotting analysis, 40% of collagen IX was the largest amount present in the pellet, the rest being soluble in the supernatant.

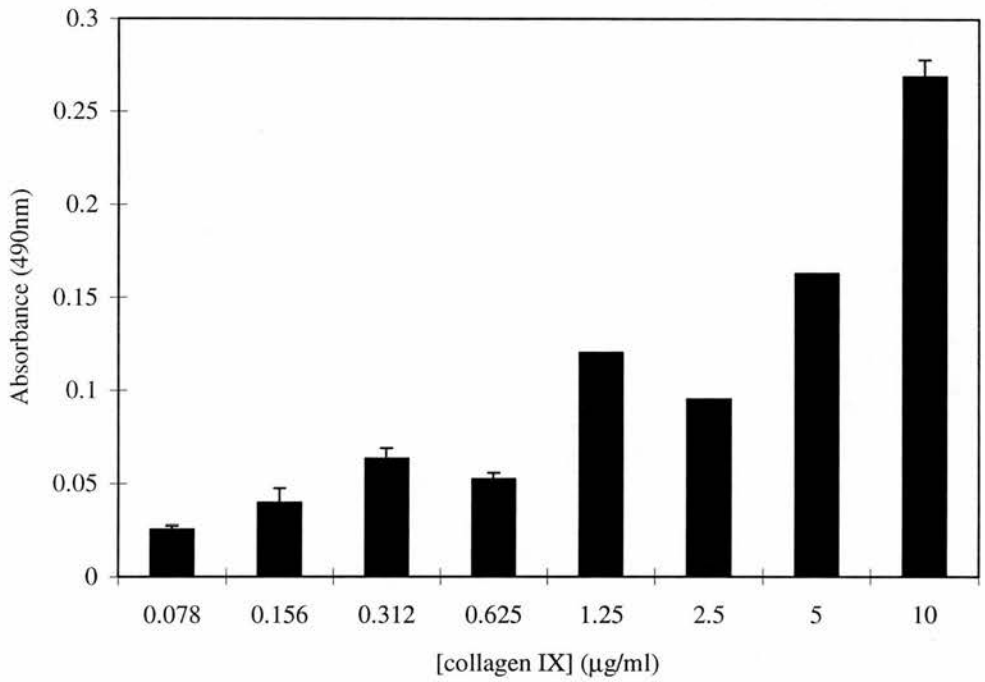


Figure 4.9. Interactions of collagen II and IX monomers. Wells coated with 1μg/ml of collagen II were incubated with increasing concentrations of collagen IX overnight. The amount of collagen IX bound was quantified by ELISA with a 1:100 dilution of affinity purified primary antibody and a 1:5000 dilution of HRP conjugated secondary antibody. Absorbance values were expressed after subtraction of controls with no collagen II and/or no collagen IX present. Error bars represent standard errors, n=3.

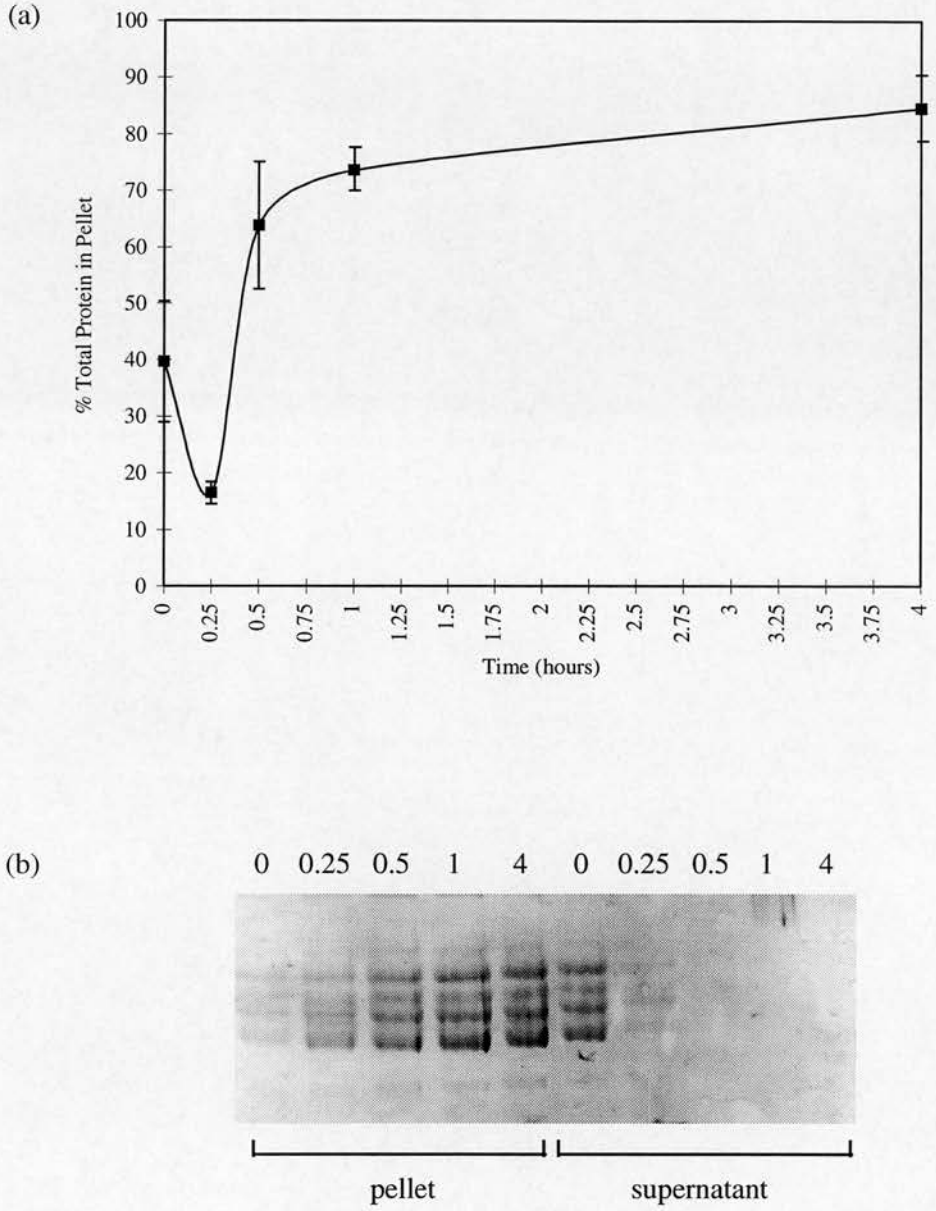


Figure 4.10. Collagen XI fibril formation. (a) kinetics of fibril formation. Collagen XI (500µg/ml in storage buffer) was mixed 1:1 with dH_2O containing 100µg/ml BSA to initiate fibril formation. At the times indicated, fibrils were collected by centrifugation and the pellet resuspended in storage buffer. Proteins in both the pellet and the supernatant were quantified by the BCA assay. Error bars represent standard error, n=3. (b) Both the pellet and the supernatant were analysed by SDS-PAGE (6% gels, reduced conditions).

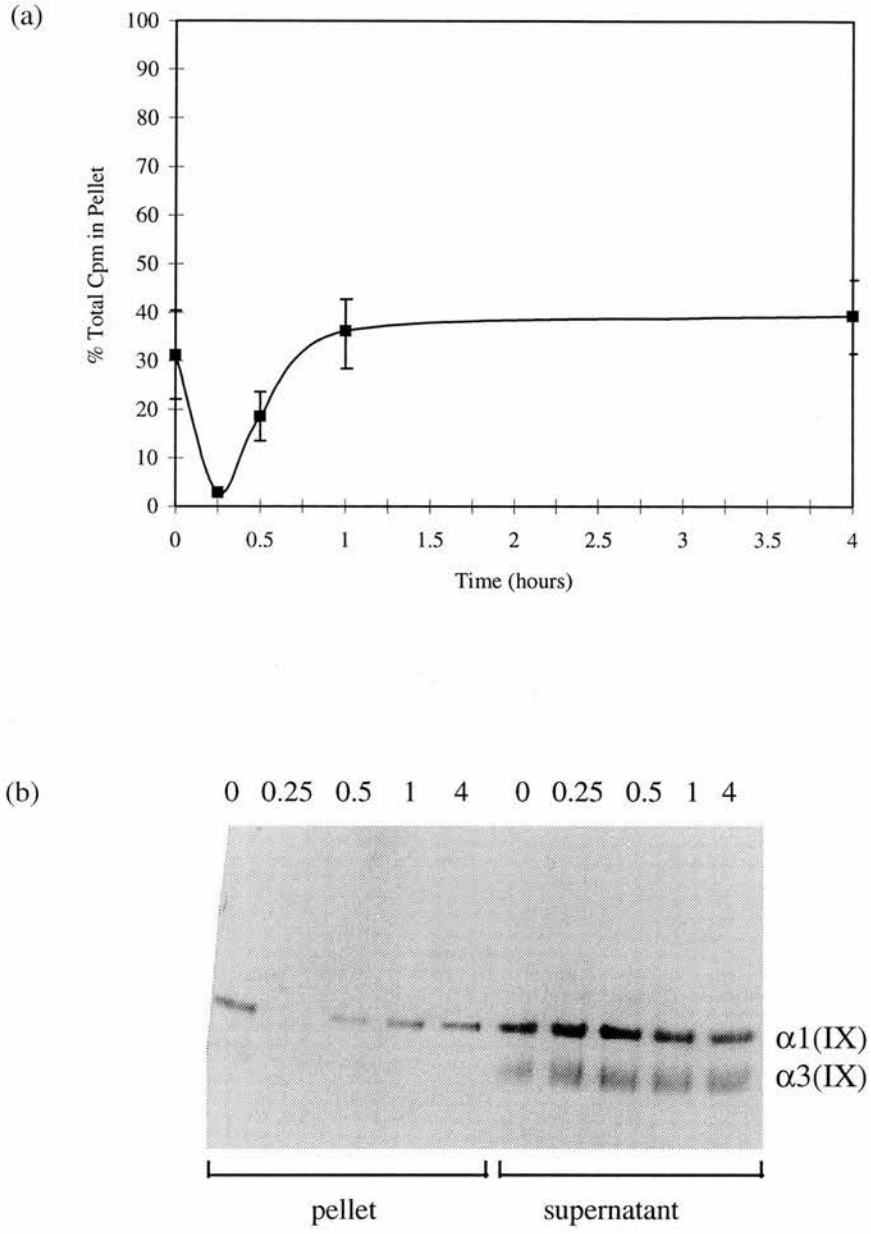


Figure 4.11. Collagen XI fibril formation in the presence of collagen IX. (a) Analysis of cpm at the given time points shows that the collagen IX in the pellet exactly mirrors the collagen XI fibril formation curve. Error bars represent the standard error, n=3. (b) Western blots using polyclonal antibodies to collagen IX diluted 1:3000 confirm that collagen IX interacts with collagen XI, although much remains soluble in the supernatant.

4.4 DISCUSSION

Compared to cartilage, the collagen II produced from chick chondrocytes cultured in alginate beads shows a large amount of the biosynthetic intermediate that retains the N-propeptide (pN II; Figure 4.4). In normal circumstances, the N-propeptide is cleaved by the enzyme N-proteinase to leave the fully processed collagen II molecule (Kadler *et al.*, 1995) but the finding that pN II represents up to 50% of the collagen II recovered (Figure 4.5) suggests that the N-proteinase enzyme is partially inhibited in this culture system. The question therefore arises as to what causes this inhibition.

Calcium and zinc ions are known to be essential for N-proteinase activity (Colige *et al.*, 1997). Removal of these ions by metal chelators, such as EDTA, has been shown to inhibit N-proteinase activity (Tuderman and Prockop, 1982; Kadler *et al.*, 1995). Considering that alginate requires the presence of calcium for polymerisation to occur (Guo *et al.*, 1989), it is probable that the alginate is sequestering most of the divalent cations present in the culture medium and thus making them unavailable for the active site of the N-proteinase. Indeed, preliminary experiments indicated that addition of 50μM $ZnSO_4$ to the culture medium increased processing of the pN II (data not shown), although it should be noted that an adequate control was not used for comparisons.

Other explanations, however, also exist. For example, since N-proteinase is a neutral metalloproteinase (Kadler *et al.*, 1995) and as the local pH within the bead may be acidic (section 3.4.2.1), this may reduce N-proteinase activity. Low pH (<6.5) conditions have been shown to reduce processing of the N-terminal domain of procollagen (Bard *et al.*, 1993), thus even if the local pH within the bead is pH 6.5 this would account for the elevated levels of pN II present in this culture system.

4.4.1 Methods of Removal of pN II

As fully processed collagen II was required for *in vitro* interaction studies, methods for removal of collagen II molecules retaining the N-propeptide were investigated. The first method attempted was to change the culture medium. Serum-free

on chondrocyte differentiation without interference by undefined components of the media (Bruckner *et al.*, 1989). The presence of low concentrations of FBS in the media was shown to cause cells to become hypertrophic and therefore stop proliferating, whereas the inclusion of insulin was found to cause chondrocyte proliferation (Bohme *et al.*, 1992; Tschan *et al.*, 1993). As FBS contains proteinase inhibitors that would stop N-proteinase activity (Pesciotta *et al.*, 1982), serum-free cultures were adopted and it was found that the pN II concentration reduced to 10% (Figure 4.5). There was, however, still some collagen II molecules that retained the N-propeptide and this was deemed unsuitable for *in vitro* interaction studies. Also, total collagen production was decreased by 30% so other methods of removal of collagen II molecules containing the N-propeptide were investigated.

It was shown that processed collagen II molecules could not be fully separated from pN II by ion exchange chromatography which was surprising. The poor separation could be due the differential expression of the N-propeptide by alternative splicing of the procollagen II mRNA (Ryan and Sandell, 1990; Sandell *et al.*, 1991). Two forms of procollagens exist, IIA and IIB (section 1.3.3.1) which include or exclude the cysteine-rich domain of the N-propeptide, respectively. Procollagen IIA is produced by fibroblast-like cells in the superficial zones of cartilage whereas procollagen IIB is the major form of collagen II produced by chondrocytes (Sandell *et al.*, 1991). As procollagen I can be purified by ion exchange chromatography, it was assumed that procollagen IIA also could be due to the high similarities of the genes encoding these two collagen types. The lack of a major proportion of the N-terminal domain in procollagen IIB however, may mean that this isoform does not carry a charge on its propeptide and therefore cannot be purified by ion exchange chromatography. As procollagen IIB is produced specifically by chondrocytes, and not by the fibroblast-like cells from the superficial zone, confirms once again that the chondrocytes in this culture system remain phenotypically stable (Figure 3.2).

This technique, even had it worked, would have had the major disadvantage that 50% of collagen II molecules would be removed and thus the N-proteinase enzyme was employed to cleave the N-propeptide from pN II, so providing 100% of fully processed collagen II molecules.

4.4.2 N-proteinase Activity

Several factors need to be considered to ensure full activity of N-proteinase *in vitro*. In addition to the requirement for divalent cations, correct conformation of the cleavage site is also important as the cleavage of heat-denatured procollagen is slower than that of native procollagen and the addition of the thiol reagent, dithiothreitol, arrests N-proteinase activity (Tuderman and Prockop, 1982; Tanzawa *et al.,* 1985). The length of the collagen triple-helical domain is also significant as the longer the α-helical domain, the faster the cleavage of the N-propeptide (Dombrowski and Prockop, 1988).

The 107kDa form of N-proteinase, purified from bovine skin, was found to have full activity on a procollagen I substrate (Colige *et al.*, 1995) but was not active on purified pN II (Figure 4.6a-c). It was, however, active on pN II present in a crude mixture of proteins from alginate bead culture (Figure 4.6d) and there are a number of possible explanations for this observation:

- The N-proteinase was purified from bovine skin and therefore may not have activity on collagen II from an avian source as the ECMs of different species (for example, mammalian and avian) show a certain degree of diversity (Elima and Vuorio, 1989). This was not however the case in this instance as the enzyme was active on pN II present in a crude mixture of proteins. Moreover, the other N-proteinases described to date have been shown to be active on procollagen from different species, for example, N-proteinase isolated from chick tendon is active on both human and bovine procollagens which implies N-proteinases are not species specific (Dombrowski and Prockop, 1988; Hojima *et al.*, 1994b).
- This N-proteinase (107kDa) is collagen I specific. Again, this was shown to be contradicted by its activity on pN II present in a mixture of proteins. Moreover, previous forms of N-proteinase, purified from skin and tendon have been shown to be active on both procollagens I and II suggesting that N-proteinases cleave the N-propeptides from both these collagen types (Tuderman *et al.*, 1978; Hojima *et al.*, 1989; Hojima *et al.*, 1994b).

- The conformation of the pN II was incorrect as N-proteinase is only active on procollagen that is in its native conformation (Tuderman and Prockop, 1982; Tanzawa *et al.*, 1985). During the purification process there may have been denaturation of the pN II through heat or proteolytic degradation but this is unlikely as each step was carried out at 4 °C and proteinase inhibitors were present at all times. Alternatively, the pN II produced in this culture system may contain 3 pools of molecules that contain (a) propeptides on all three α-chains, (b) propeptides on 2 α-chains or (c) a propeptide on only one of the three α-chains. It is possible that the enzyme requires all three propeptides to provide the correct conformation for activity and this population represents a small percentage of the 3 pools of molecules present.

- N-proteinase requires a co-factor for activity on a collagen II substrate. Due to the cleavage of pN II present in a mixture of proteins, it can be concluded that the co-factor is produced by chondrocytes cultured in alginate beads. Investigations into the identity of this co-factor were unsuccessful, but collagens IX, XI and XIV can be ruled out (Figure 4.7a and b). These experiments presumed that the substrate was either in the wrong conformation (collagens IX and XI were required to form a heterotypic aggregate which would place pN II in the correct conformation for N-proteinase activity) or that another molecule was required to direct the N-proteinase to the correct docking site on the pN II molecule (collagen XIV).

- The co-factor may be another enzyme that processes the N-proteinase to increase its specificity for pN II or conversely, the N-proteinase may activate the co-factor that could represent another enzyme which would then cleave pN II. Alternatively, the co-factor could change the conformation of the N-proteinase and/or the pN II.

- Although the identity of the potential co-factor was unknown, it may be present on the cell membrane as the crude extract of proteins from alginate bead culture also contained chondrocytes. Investigations into its identity would then have to be extended to integrins, transmembrane collagens, membrane-anchored proteoglycans or annexins (Loeser, 1993; Rehn and Pihlajaniemi, 1996; Redini *et al.*, 1997; Von der Mark and Mollenhauer, 1997).

4.4.3 Interactions of Cartilage Collagens

The interactions of cartilage collagens, at both the monomer and the fibril levels, were investigated (Figures 4.9, 4.10 and 4.11). It was found by the ELISA technique that collagens II and IX interact at the monomer level (Figure 4.9). It was assumed that this result was not an artefact due to the presence of collagen II fibrils, as the collagen was kept in low concentrations, at 4 °C in high ionic strength buffer which inhibits fibril formation (Wood and Keech, 1960). This result indicates that collagen II fibrils are not a prerequisite for the attachment of collagen IX molecules and that there may be a nucleation event during the formation of heterotypic cartilage fibrils which would require at least 2 collagen types to interact prior to fibril growth. In support of this nucleation-growth model are the conjectures of Silver *et al.* (1992) where a nucleus forms at each end of a growing fibril and growth of the fibril then proceeds by propagation of the two structural nuclei.

The finding that collagens II and IX interact at monomer level also suggests that collagen IX could limit the diameter of the collagen II fibrils from the start of fibril formation (section 1.3.4; Duance *et al.*, 1990). Also, in regard to the SLS aggregates present in alginate bead culture, the interactions of collagens II and IX supports the theory that SLS aggregates are comprised of these 2 collagen types (section 3.4.2.2). ELISA assays of other combinations of cartilage collagens (that is, collagens II and XI or collagens IX and XI) were also investigated but these were unsuccessful due to cross-reactivity of the polyclonal antibodies. In the case of collagens II and XI, this is not surprising as the α3(XI) chain is an overglycosylated form of the α1(II) chain (section 1.3.3.2; Reese and Mayne, 1981; Bernard *et al.*, 1988).

The interactions of collagens IX and XI during fibril formation also indicates a nucleation event in the formation of heterotypic cartilage fibrils. In this experiment, the interaction of collagens IX and XI was assumed to be due to the N-terminus of collagen XI interacting with some undefined region on the collagen IX molecule. This undefined region could be the GAG chain as this negatively charged area of the molecule could interact with the positively charged PARP domain on the N-terminus of collagen XI (section 1.3.3.2). It should be noted that purification of collagen IX by

molecule could interact with the positively charged PARP domain on the N-terminus of collagen XI (section 1.3.3.2). It should be noted that purification of collagen IX by ion exchange chromatography selectively purifies the proteoglycan form of collagen IX and that it was this form which was used in both the ELISA assay and the *in vitro* reconstitution experiments. It has been proposed that the GAG chain may guide collagen IX to the correct docking site on the surface of the collagen II fibril (Diab *et al.*, 1996) and thus the interactions of collagens II and IX at the monomer level may also be due to the presence of the GAG chain on collagen IX. The role of the GAG chain in both these interactions could be elucidated by experiments where the collagen IX was incubated with chondroitinase ABC prior to fibril formation/monomer binding.

The interaction of collagens IX and XI may be an artefact due to the presence of residual collagen II. This is difficult to assess as on SDS-PAGE the α1(II) chains co-migrate with α3(XI) chains and due to the high similarity of these 2 collagen chains it is difficult to detect the presence of collagen II immunologically. To confirm that collagen II was not present, monoclonal antibodies would have to be made against particular regions of the collagen II molecule that differ from collagen XI. It should be noted however that the α1(XI), α2(XI) and α3(XI) bands are present as a 1:1:1 ratio as quantified by densitometry (Figure 4.10b), and this ratio shows that there is no increase in the intensity of the α3(XI) and thus it is unlikely that residual α1(II) chains were present.

The highest amount of collagen IX present in the pellet in these experiments was 40% (Figure 4.11a) and the reason for the remainder being soluble in the supernatant may be due to saturation of the binding sites on the collagen XI fibrils. Fibril formation with an increased concentration of collagen XI monomers, or a decrease in collagen IX molecules would clarify this. Alternatively, if residual collagen II was present, the amount of collagen IX binding to the collagen XI fibrils would be regulated by the amount of collagen II present.

4.4.4 Final conclusions

This chapter examined methods of removal of the high amount of pN II produced by chick chondrocytes cultured in alginate beads and investigated the interactions of collagen IX with collagens II and XI at both monomer and fibril levels.

It was shown that the pN II biosynthetic intermediate was extremely difficult to remove. The results of serum-free alginate bead cultures were encouraging as 90% of pN II molecules were removed but total collagen production was also reduced. Addition of the N-proteinase enzyme to pN II present in a crude mixture of proteins resulted in complete processing of the pN II and was the only assay that showed N-proteinase activity. The addition of this enzyme to the crude mixture of proteins at the initial stages of collagen II purification would result in large amounts of the fully processed form of collagen II, but the N-proteinase enzyme is difficult to purify in the large quantities that would be needed.

Investigations into the interactions of collagen IX with collagens II and XI found that collagen II fibrils are not a prerequisite for the attachment of collagen IX molecules and that the interaction of these collagen types at monomer level indicates a nucleation event in the assembly of a heterotypic cartilage fibril. Collagens IX and XI were found to interact during *in vitro* reconstitution experiments and thus all three of these collagen types may interact to form a nucleation aggregate.

Chapter 5

Final Discussion

This thesis set out to address the question "*Do alginate beads provide an environment similar to that of cartilage for the culture of chick chondrocytes*?". This question was examined using a range of biochemical and other techniques to compare the ECM constituents produced in alginate beads to those observed *in vivo*. The major results presented are:

- cartilage-specific collagens (II, IX, X and XI) are produced in a similar ratio to those found *in vivo* (Chapter 2)
- collagen IX is present in the medium as both a proteoglycan and a non-proteoglycan form (Chapter 2)
- the chondrocytes remained phenotypically stable over a 14 day culture period (Chapter 3)
- SLS aggregates substitute collagen fibrils in the matrix that surrounds the chondrocytes (Chapter 3)
- a large amount of pN II collagen is produced, which cannot be processed by N-proteinase (107 kDa) unless a crude mixture of proteins is present (Chapter 4)

Based on this evidence, the following discussion considers some of the strengths and weaknesses of the use of alginate beads for the culture of chondrocytes and whether this system provides a good *in vitro* model for cartilage morphogenesis.

5.1 Strengths of Alginate Bead Culture

Chondrocytes must be cultured in suspension as they dedifferentiate and fail to produce cartilage-specific collagens when cultured in monolayer (section 2.1; Cancedda *et al.*, 1995). The traditional suspension cultures used were soft agar and agarose gels but alginate beads were introduced as an alternative as they readily dissociate in the presence of a calcium chelator to allow rapid recovery and quantitation of chondrocytes and ECM proteins (Guo *et al.*, 1989). The dissociation gives rise to two compartments: the cells with their associated matrix (CM), which represents the combined pericellular and territorial matrices, and the further-removed

matrix (FRM) which depicts the interterritorial matrix (Hauselmann *et al.*, 1994). Little is known about the relationship between chondrocytes and their surrounding matrix compartments and the alginate bead culture system thus provides an *in vitro* method to investigate the influence of chondrocytes on the organisation and turnover of these matrices.

Turnover of ECM components *in vivo* is difficult to study and has given rise to differing half-lives of collagens and proteoglycans (Hardingham *et al.*, 1991; Hauselmann *et al.*, 1996) and therefore *in vitro* cell culture techniques have been adopted to provide a better understanding of ECM turnover. For the study of cartilage turnover this has proved difficult as suspension cultures are required to maintain chondrocyte phenotype. Most suspension cultures, for example agarose gels, cannot be used to study ECM turnover as they do not readily dissociate and thus overnight extraction in dissociative conditions (for example, guanidine hydrochloride) is required to release ECM components (Delbruck *et al.*, 1986). Due to the dissociation of alginate beads in the presence of a calcium chelator, cells and ECM components can easily be collected and therefore cartilage turnover *in vitro* studied without difficulty. Proteoglycan turnover in alginate beads has been well documented but few studies investigate collagen turnover (Hauselmann *et al.*, 1992; Grandolfo *et al.*, 1993; Mok *et al.*, 1994).

A further advantage of alginate beads is that collagens II and IX are present in large quantities in the medium, from where they can easily be purified (Figure 2.3). Other studies, for example, fibrillogenesis assays require collagens with telopeptides on each α-chain (McPherson *et al.*, 1985) and therefore extraction of collagens from tissues by pepsin digestion is not possible as the telopeptides are removed. Thus culture systems and recombinant expression of collagens (Fertala *et al.*, 1994) have been introduced as vehicles for the production of collagens for fibrillogenesis studies. Alginate beads are especially useful for the production of collagens II and IX, as these are released into the medium from where they can be easily purified. As these collagens are continually released, this culture system represents a technique for the long-term production of collagens II and IX. Moreover, this is only true of this suspension culture system as the medium of agarose gels do not contain measurable

levels of collagens at any time (Mr Ulrich Blaschke, Munster, Germany, personal communication). Also, the large amount of collagen IX produced is especially advantageous as this collagen type comprises a relatively small amount (10%) of total collagen *in vivo* (Vaughan *et al.*, 1988).

The alginate bead culture system is also useful in the study of chondrocyte differentiation *in vitro*, as little is known about the factors that control this phenomena *in vivo* (Cancedda *et al.*, 1995). The use of serum-free cultures, where specific factors are added to the medium, allows the effects of interleukins, growth factors and other molecules on chondrocyte differentiation to be assessed (Bruckner *et al.*, 1989; Tschan *et al.*, 1990; Bohme *et al.*, 1992). These experiments have been extended to chondrocytes derived from the different zones of articular cartilage as the cells retain the characteristics of the zone from which they were derived (Aydelotte *et al.*, 1986; Bohme *et al.*, 1995). Further analysis of serum-free cultures containing different combinations of growth factors and other molecules will eventually lead to understanding of all the factors that control differentiation *in vivo*.

5.2 Weaknesses of Alginate Bead Culture

This study also shows weaknesses in the use of alginate beads for the culture of chondrocytes. The 2 main uses of culture systems are to provide (1) a model system for the study of cartilage morphogenesis and (2) a vehicle for the production of cartilage collagens for use in other studies. This culture system is not suitable for the study of cartilage morphogenesis as alginate disrupts normal fibril assembly. SLS aggregates were found to substitute fibrils in both the CM and FRM (Figures 3.7 and 3.8), although the cause of these specific aggregations was unknown. It was suggested that it may be due either to the charge on the alginate, the high amount of pN II present (see below) or the local acidity within the bead, all of which are known to affect collagen fibril assembly *in vitro*. For instance, increasing pN-collagen content has been found to induce pleomorphism in the assembly of collagen fibrils (Hulmes *et al.*, 1989; Mould *et al.*, 1990) while acidity, caused by the presence of highly sulphated GAGs, has led to the formation of SLS in limb bud cultures (Merker

et al., 1978). Although SLS have been proposed to be a nucleation event in fibril assembly (Bruns *et al.*, 1979), in this culture system there was no further fibril growth and therefore alginate beads remain a poor model for *in vitro* cartilage morphogenesis studies.

Another disadvantage of the alginate bead system is that a large amount of the collagen II produced was not fully processed and retained the N-propeptide (Figure 4.5). In addition to the requirement for fully processed collagen II for *in vitro* interaction studies, the persistence of the N-propeptide on collagen II has the additional disadvantage in that it causes feedback inhibition of collagen synthesis (Weistner *et al.*, 1979; Wu *et al.*, 1986). Methods of removing or processing this biosynthetic intermediate were investigated but proved difficult. Serum-free cultures were attempted but, while the results were encouraging, did not lead to complete processing of pN II molecules (Figure 4.5) and ion exchange chromatography was shown to ineffective in the removal of collagen II molecules that retained the N-propeptide (Figure 4.2). The N-proteinase enzyme was employed to cleave the N-propeptide and thus leave fully processed collagen II molecules and although it was unsuccessful on a purified pN II substrate, the enzyme was active on pN II present in a mixture of proteins from alginate bead culture (Figure 4.6). To produce a large amount of fully processed collagen II, the N-proteinase could be added to the crude mixture at the initial stages of collagen purification, but this approach would require large quantities of the enzyme which is relatively difficult to produce (Colige *et al.*, 1995). Also, less cartilage collagens would be synthesised in the first place due to the feedback inhibition of collagen synthesis exerted by the uncleaved N-propeptide. Therefore, the use of this culture system for the production of cartilage collagens for other studies also has its drawbacks.

Also, although alginate beads provide a suitable environment for chondrocytes to maintain phenotype, this is very different from what the cells experience *in vivo*. For example, the chondrocytes do not suffer the mechanical forces present in cartilage, there is not the segregation of differentiated chondrocytes like that observed *in vivo* and the culture systems do not simulate the gradient of nutrients/pH/growth factors/etc found in cartilage. There are also major differences in the ionic nature of

each environment. Therefore chondrocytes in suspension cultures are grown in a synthetic environment that is vastly different from that provided by cartilage and thus any results obtained must be analysed with these considerations in mind.

5.4 Future Work

Although this study provides answers to some specific questions concerning alginate bead culture of chick chondrocytes, there are still a number of questions that need to be addressed:

- SLS aggregates were found to substitute banded collagen fibrils in both the CM and FRM matrices (Figures 3.7 and 3.8) but it was unclear as to which collagens comprised these aggregates. Immunolabelling experiments with antibodies to collagens II, IX, X and XI would clarify this while analysis of normal and diseased cartilages would elucidate whether SLS occur *in vivo* like some reports have suggested (Perez-Tamayo, 1972; Cho and Garant, 1981). The role of these specific aggregates would then become clearer.
- The reason why the 107kDa form of N-proteinase did not cleave the N-propeptide from a purified pN II substrate, but was active when a mixture of proteins was present, was unresolved. It was proposed that a co-factor was required and the presence of a potential co-factor could be investigated by fractionating the crude extract and adding the proteins present in each fraction to the N-proteinase assay. If any of these fractions was found to induce activity of the N-proteinase on a purified pN II substrate, subsequent identification of the co-factor would elucidate whether it was an ECM component or a integral part of the cell membrane. Alternatively, it was proposed that the pN II substrate was present in the incorrect conformation. To elucidate whether this was true, a different form of N-proteinase, for example the one described by Hojima *et al.* 1989 that has previously been shown to have full activity on a collagen II substrate, could be added to purified pN II.

- serum-free cultures and preliminary experiments involving the addition of zinc to the culture medium were shown to increase the processing of pN II to collagen II. Further investigations into the addition of growth factors and interleukins to the medium of alginate bead culture would eventually allow the reproduction of *in vivo* conditions. This would have the additional advantage that inhibition of collagen synthesis by persistence of the N-propeptide would be abolished and thus there would be a higher rate of collagen production. Indeed, it has recently been shown that elevated levels of zinc ions selectively promote the production of collagen (Koyano *et al.*, 1996). The effect of the addition of βAPN to the culture medium needs to be more closely examined and its addition to this culture system re-evaluated.
- preliminary interaction studies indicate the formation of a nucleation aggregate during the initial stages of assembly of a heterotypic cartilage fibril. Rotary shadowing experiments could confirm the presence of these assemblies while ELISA assays with other combinations of collagen types, using monoclonal antibodies to ensure there was no cross-reactivity, would also confirm interactions of the collagen types at monomer level.

5.5 Final Conclusions

The alginate bead system for the culture of chick chondrocytes provides an environment like that of cartilage, in that the cells remained phenotypically stable and produced cartilage-specific collagens in similar ratios to those *in vivo*. There were, however, 2 major differences in the ECM produced here compared to that of cartilage; no banded collagen fibrils were present and there was a large amount of the biosynthetic precursor of collagen II that retained the N-propeptide. Therefore, alginate beads provide a powerful technique for the study of chondrocyte differentiation *in vitro* and for the production of large quantities of collagen IX, but other approaches must be taken to study cartilage morphogenesis and to produce large quantities of fully processed collagen II for use in other studies.

Appendix

General Methods

A.1 SDS-PAGE

The method developed by Laemmli (1970) was used for discontinuous SDS-PAGE analysis of proteins. For more detailed procedures on preparation of gels and samples for electrophoresis see Hames (1990).

6% (w/v) acrylamide/0.32% (w/v) bisacrylamide gels were used for separation of proteins with molecular weights in the range 60kDa to 200kDa. For SDS-PAGE under reducing conditions, sample buffer contained 3% (v/v) β-mercaptoethanol whereas under non-reducing conditions water substituted the β-mercaptoethanol. A constant electric current (45mA per gel) was applied to gels in a vertical gel electrophoresis apparatus (LKB) cooled with water, for approx. 3 hours until the tracking dye front was 1cm from the bottom of the gel. Gels were either taken for western blotting (section A.3) or fixed in 45% (v/v) methanol, 10% (v/v) acetic acid for 1 hour before staining or fluorography. In some cases mini-gels were run as above using a "tall mighty small" (Hoefer) electrophoresis unit, where run and staining times were decreased.

A.2 GEL STAINING METHODS

A.2.1 Coomassie blue staining

Gels were stained with 0.25% (w/v) Coomassie Brilliant Blue R-250 in 45% (v/v) methanol, 10% (v/v) acetic acid for 15 to 20 hours, with constant shaking on a Denley reciprocal shaker. Protein bands were visualised by destaining with several changes of 7.5% (v/v) acetic acid, 5% (v/v) ethanol and this process could be speeded up by heating to 50 °C.

A.2.2 Silver Staining

Silver staining is a more sensitive method than Coomassie blue for staining SDS-PAGE gels and the following method was adapted from Morrissey (1981). Following

SDS-PAGE, gels were fixed overnight in 45% (v/v) methanol, 10% (v/v) acetic acid and then rehydrated by addition of dH_2O for 1 hour. The proteins were subsequently reduced by incubation for 2 hours in dH_2O containing 5µg/ml dithiothreitol. After removal of this solution, 0.1% (w/v) silver nitrate was added for a further 2 hours followed by washing with dH_2O. Protein bands were visualised by addition of 3% (w/v) sodium carbonate containing 100µl of 40% formaldehyde and the gels were agitated in this solution until the desired level of staining was reached. The reaction was then stopped with 2.3M citric acid and after approx. 30 minutes, the gel was placed in 2% (v/v) glycerol.

A.2.3 Fluorography

For the detection of radiolabelled proteins, fluorography was carried out. Following SDS-PAGE, gels were fixed for at least 1 hour in 45% (v/v) methanol, 10% (v/v) acetic acid and then rehydrated by addition of dH_2O for 1 hour. "Amplify" (Amersham) was added to the gels (50ml per gel) and left for 30 minutes with constant shaking on a Denley reciprocal shaker. The gels were immediately placed onto a piece of Whatman filter paper (3MM) and dried down on a gel dryer. Fluorograms were exposed to either XAR or Biomax MR scientific imaging film (Kodak) at -70 °C in a X-omatic cassette. Times of exposure varied but where direct comparisons were to be made exposure times were identical.

A.3 Western Blotting

Immunological detection of proteins by electrophoretic blotting onto nitro-cellulose was performed using the method developed by Towbin *et al.* (1979).

A.3.1 Transfer of Proteins to Nitrocellulose

Following SDS-PAGE, gels were removed from the electrophoresis apparatus and placed in blotting buffer (20mM Na_2HPO_4, 20% (v/v) methanol, pH 9.4) for 1-2

minutes. Methanol is present in this buffer to strip the SDS from the proteins which functions both to renature them and to ensure that they will adhere to the nitrocellulose membrane. The bottom right hand corner of each gel was removed for later identification of orientation. The gels were then "sandwiched" in the open clamp assembly of a Biorad TRANS-BLOT cell, ensuring that there were no air bubbles trapped and that both the gel and nitrocellulose were wet at all times. The order of the "sandwich" was a scotch-brite pad, followed by a piece of 3MM Whatman filter paper cut to the size of the gel, the gel itself, a piece of 0.2μm nitrocellulose membrane the same size as the filter paper (Sartorius, Epsom, Surrey; bottom right hand corner removed), a second piece of filter paper and finally a second scotch-brite pad. The clamp assembly was placed into a transfer tank containing blotting buffer ensuring that the gel was nearest to the negative electrode. Transfer of proteins from the gel to the nitrocellulose was completed by running a current of 250mA (maximum voltage 20V) through the apparatus for approx. 16 hours.

A.3.2 Detection of Proteins by Enhanced Chemiluminescence (ECL)

To ensure the transfer of proteins to the nitrocellulose was complete, 0.4% (w/v) Ponceau S in 3% (w/v) TCA was added to the membrane for 1 minute before washing with several changes of dH_2O to allow the protein bands to be visualised. In all subsequent steps the nitrocellulose membrane was kept wet at all times while agitation of the membrane ensured extensive washing at each step. All procedures were carried out at room temperature.

Unspecific sites on the membrane were blocked by the addition of 5% (w/v) dried milk or 0.5% BSA in TBST (50mM Tris-HCl, 0.15M NaCl, 0.05% (v/v) Tween-20, pH 7.9) for 1 hour. After washing with TBST (1x15 mins and 2x5 mins), primary antibody (diluted with TBST) was added to the membrane for 90 minutes. The TBST washing step was repeated and horseradish peroxidase conjugated anti-rabbit IgG second antibody (dilutions stated in main text) was then added for a further hour. Following a further TBST wash step, the membrane was incubated in ECL reagents

(Amersham) mixed in a 1:1 ratio for exactly 1 minute. The membrane was then placed onto a piece of Whatman 3MM filter paper, covered with cling film, placed in a Kodak X-Omatic cassette and exposed immediately to Kodak X-OMAT AR scientific imaging film. Exposure times depended on the amount of antigen present and dilution of the primary antibody.

A.3.3 Stripping of Antibodies from Nitrocellulose Membranes

Western blots could be stripped and reprobed using a modification of the method of Kaufman *et al.* (1987). Stripping buffer (100mM β-mercaptoethanol, 2% (w/v) SDS, 62.5mM Tris-HCl, pH 6.7) was incubated with the nitrocellulose membrane at 50 °C for 30 minutes. Following extensive washing with TBST (1x15 mins, 1x10 mins, 1x5 mins), the membrane was exposed to Kodak X-OMAT AR scientific imaging film for 30mins to ensure complete removal of the antibody. The western blotting procedure was then repeated from the initial blocking step with a new primary antibody.

A.3.4 Affinity Purification of Antiserum

Affinity purification eliminates a lot of cross-contamination of polyclonal antibodies that is usually observed. Partially purified antigen (either collagen II or IX) was loaded into a single well spanning the width of a 6% acrylamide gel, and was subject to SDS-PAGE (section A.1). Proteins were transferred to nitrocellulose as described previously (A.3.1) and protein bands were visualised with 0.4% (w/v) Ponceau S. The strip of nitrocellulose corresponding to the antigen of interest was excised and specific marks made to allow identification of the antigen face. Unspecific sites of the membrane were blocked with TBS (50mM Tris-HCl, 0.15M NaCl, pH 7.4) containing 3% (w/v) BSA for 90 minutes with shaking. After extensive washing in TBS (1x15 mins, 2x5 mins), the strip was placed on Nescofilm (antigen side up) in a humidity chamber.

Antiserum (~250µl) was placed on the strip and incubated at room temperature for 2 hours with gentle shaking. Following removal of excess serum, the strip was washed with TBST (1x15 mins, 2x5 mins, 250µl each wash) before bound antibody was eluted from the strip by addition of 250µl of 0.2M glycine-HCl, pH 2.8 for 20 minutes with gentle shaking. This buffer was removed and immediately neutralised with an equal volume of 0.1M Tris-HCl, pH 8.5. The elution and neutralisation steps were repeated twice to remove as much affinity purified antibody as possible. Affinity purified antibodies were stored at 4 °C, in the presence of 0.1% (w/v) sodium azide. Following washing in TBST, the affinity strip was stored at 4 °C in TBS containing 0.1% (w/v) sodium azide for re-use.

A.4 Densitometry

Proteins separated by SDS-PAGE were quantified by scanning Coomassie stained gels or fluorographs in a Joyce-Loebl Chromoscan 3 coupled to a DCS microcomputer for digital analysis. The chromoscan was operated in absorbance mode with an aperture of 0.3 x 5mm. Scans were carried out with a red (626nm) filter and a 100 W tungsten halogen lamp as a light source. Before calculating the integrated peak areas, a background subtraction was made. The relative molar percentage of each collagen chain was determined and the different molecular weights were accounted for as described previously (section 2.2.5).

A.5 Bicinchoninic Acid (BCA) Protein Assay

Protein concentrations were measured using the BCA protein assay (Pierce) which utilises the ability of proteins to reduce Cu^{2+} to Cu^{1+} in alkaline conditions. Cu^{1+} is subsequently detected by the reagent BCA. The microtitre plate protocol was used with standards in the range 0 to 250µg/ml collagen I, and all samples were run in duplicate. Working reagent was prepared by mixing 50 parts solution A (containing the BCA detection reagent) with 1 part solution B (4% copper sulphate). 10µl of each standard, blank or sample was placed in the appropriate microtitre plate well and

200µl of the working reagent was added. The plate was then covered and incubated at 60 °C for 30 minutes. After cooling to room temperature, the absorbance at 490nm was measured in a Dynatech MR7000 microplate reader. Protein concentrations of unknown samples were calculated automatically from the standard curve of varying collagen I concentrations using Dynatech Data Reduction software.

REFERENCES

Adachi, E. & Hayashi, K. *In vitro* formation of fine fibrils with a D-periodic banding pattern from type V collagen. (1985) Collagen Rel. Res **5**, 225-232

Adachi, E. & Hayashi, T. *In vitro* formation of hybrid fibrils of type V collagen and type I collagen - limited growth of type I collagen into thick fibrils by type V collagen. (1986) Conn. Tiss. Res. **14**, 257-266

Adam, M. & Deyl, Z. Altered expression of collagen phenotype in osteoarthritis. (1983) Clin. Chim. Acta **133**, 25-32

Ahmad, N.N., Dimascio, J., Knowlton, R.G. & Tasman, W.S. Stickler syndrome: a mutation in the nonhelical 3' end of type II procollagen gene. (1995) Arch. Opthalmol. **113**, 1454-1457

Aigner, T., Bertling, W., Stoss, H., Weseloh, G. & von der Mark, K. Independent expression of fibril-forming collagens I, II, and III in chondrocytes of human osteoarthritic cartilage. (1993) J. Clin. Invest. **91**, 829-837

Ala-kokko, L., Baldwin, C.T., Moskowitz, R.W. & Prockop, D.J. Single base mutation in the type II procollagen gene (COL2A1) as a cause of primary osteoarthritis associated with a mild chondrodysplasia. (1990) Proc. Natl. Acad. Sci. USA **87**, 6565-6568

Ayad, S., Marriott, A., Morgan, K. & Grant, M.E. Bovine cartilage types VI and IX collagens: characterisation of their forms *in vivo*. (1989) Biochem. J. **262**, 752-761

Ayad, S., Marriott, A., Brierly, V.H. & Grant, M.E. Mammalian cartilage synthesises both proteoglycan and non-proteoglycan forms of type IX collagen. (1991) Biochem. J. **278**, 441-445

Ayad, S., Boot-Hanford, R.P., Humphries, M.J., Kadler, K.E. & Shuttleworth, C.A. (1994) "The Extracellular Matrix Facts Book". Academic Press, Ltd., London

Aydelotte, M.B., Schleyerbach, R., Zeck, B.J. & Kuettner, K.E. (1986) in "Articular Cartilage Biochemistry" (Kuettner, K., Schleyerbach, R. & Hascall, V.C. eds.), Articular chondrocytes cultured in agarose gel for study of chondrocyte chondrolysis. pp. 235-256, Raven Press, Ltd., New York

Aydelotte, M.B., Greenhill, R.R. & Kuettner, K.E. Differences between sub-populations of cultured bovine articular chondrocytes. II. Proteoglycan metabolism. (1988) Conn. Tiss. Res. **18**, 223-234

Aydelotte, M.B. & Kuettner, K.E. (1993) in "Joint Cartilage Degradation: Basic and Clinical Aspects" (Woessner, J.F. & Howell, D.S. eds.), Heterogeneity of Articular Chondrocytes and Cartilage Matrix. pp. 37-65, Marcel Dekker, Inc., New York

Bard, J.B.L., Hulmes, D.J.S., Purdom, I.F. & Ross, A.S.A. Chick corneal development *in vitro*: diverse effects of pH on collagen assembly. (1993) J. Cell Sci **105**, 1045-1055

Benya, P.D. & Schaffer, J.D. Dedifferentiated chondrocytes reexpress the differentiated collagen phenotype when cultured in agarose gels. (1982) Cell **30**, 215-224

Berg, R.A. Determination of 3- and 4-hydroxyproline. (1982) Meth. Enzymol. **82**, 372-398

Bernard, M., Yoshioka, H., Rodriguez, E., van der Rest, M., Kimura, T., Ninomiya, Y., Olsen, B.J. & Ramirez, F. Cloning and sequence of Pro-α1(XI) collagen cDNA demonstrates that type XI belongs to the fibrillar class of collagens and reveals that the expression of the gene is not restricted to cartilaginous tissue. (1988) J. Biol. Chem. **263**, 17159-17166

Birk, D.E., Fitch, J.M., Babiarz, J.P. & Linsenmayer, T.F. Collagen type I and type V are present in the same fibril in the avian corneal stroma. (1988) J. Cell Biol. **106**, 999-1008

Birk, D.E., Zycband, E.I., Winkelmann, D.A. & Trelstad, R.L. Collagen fibrillogenesis *in situ*: fibril segments are intermediates in matrix assembly. (1989) Proc. Natl. Acad. Sci. USA **86**, 4549-4553

Birk, D.E., Fitch, J.M., Babiarz, J.P., Doane, K.J. & Linsenmayer, T.F. Collagen fibrillogenesis *in vitro*: interaction of types I and V collagen regulates fibril diameter. (1990) J. Cell Sci. **95**, 649-657

Blaschke, U.K., Hedbom, E. & Bruckner, P. Distinct isoforms of chicken decorin contain either one or two dermatan sulfate chains. (1996) J. Biol. Chem. **271**, 30347-30353

Bohme, K., Conscience-Egli, M., Tschan, T., Winterhalter, K.H. & Bruckner, P. Induction of proliferation or hypertrophy of chondrocytes in serum-free culture: the role of insulin-like growth factor-I, insulin or thyroxine. (1992) J. Cell Biol. **116**, 1035-1042

Bohme, K., Winterhalter, K.H. & Bruckner, P. Terminal differentiation of chondrocytes in culture is a spontaneous process and is arrested by transforming growth factor-β2 and basic fibroblast growth factor in synergy. (1995) Exp. Cell Res. **216**, 191-198

Bonaldo, P., Russo, V., Bucciotti, F., Doliana, R. & Colombatti, A. Structural and functional features of the alpha-3 chain indicate a bridging role for chicken collagen VI in connective tissues. (1990) Biochem. **29**, 1245-1254

Bonaventure, J., Kadhom, N., Cohen-Solal, L., Ng, K.H., Bourguignon, J., Lasselin, C. & Freisinger, P. Reexpression of cartilage-specific genes by dedifferentiated human articular chondrocytes cultured in alginate beads. (1994) Exp. Cell Res. **212**, 97-104

Bonnucci, E., Sartori, E. & Spina, M. Osteopetrosis Fetalis: Report on a case, with special reference to ultrastructure. (1975) Virchows Arch. A. Path. Anat. and Histol. **368**, 109-121

Bounelis, P. & Daniel, J.C. The effect of ascorbate on embryonic chick sternal chondrocytes cultured in agarose. (1983) Tissue Cell **15**, 683-693

Brewton, R.G., Wright, D.W. & Mayne, R. Structural and functional comparison of type IX collagen-proteoglycan from chicken cartilage and vitreous humor. (1991) J. Biol. Chem. **266**, 4752-4757

Brewton, R.G. & Mayne, R. (1994) in "Extracellular Matrix Assembly and Structure" (Yurchenko, P.D., Birk, D.E. & Mecham, R.P. eds.), Heterotypic type II, IX and XI fibrils: comparison of vitreous and cartilage forms. pp. 129-170, Academic Press, Inc., London

Brierly, V.H., Ayad, S. & Grant, M.E. Types II, IV and IX collagens in normal and osteoarthritic human articular cartilage. (1991) Biochem. Soc. Trans. **19**, 379S

Brodsky, B. & Eikenberry, E.F. Supramolecular collagen assemblies. (1985) Ann. N. Y. Acad. Sci. **460**, 73-84

Brown, D.C. & Vogel, K.G. Characterisation of the *in vitro* interaction of a small proteoglycan (PGII) of bovine tendon with type I collagen. (1989) Matrix **9**, 468-478

Bruckner, P., Vaughan, L. & Winterhalter, K.H. Type IX collagen from sternal cartilage of chicken embryo contains covalently bound glycosaminoglycans. (1985) Proc. Natl. Acad. Sci. USA **82**, 2608-2612

Bruckner, P., Mendler, M., Steinmann, B., Huber, S. & Winterhalter, K.H. The structure of human collagen type IX and its localisation in fetal and infant cartilage fibrils. (1988) J. Biol. Chem. **263**, 16911-16917

Bruckner, P., Horler, I., Mendler, M., Houze, Y., Winterhalter, K.H., Eich-Bender, S.G. & Spycher, M.A. Induction and prevention of chondrocyte hypertrophy in culture. (1989) J. Cell Biol. **109**, 2537-2545

Bruckner, P. & van der Rest, M. Structure and function of cartilage collagens. (1994) Microsc. Res. Tech. **28**, 378-384

Bruns, R.R., Hulmes, D.J.S., Therrien, F. & Gross, J. Procollagen segment-long-spacing crystallites: their role in collagen fibrillogenesis. (1979) Proc. Natl. Acad. Sci. USA **76**, 313-317

Burgeson, R.E. & Hollister, D.W. Collagen heterogeneity in human cartilage: identification of several new collagen chains. (1979) Biochem. Biophys. Res. Comm. **87**, 1124-1131

Burgeson, R.E. (1987) in "Structure and Function of Collagen Types" (Mayne, R. & Burgeson, R.E. eds.), Type VII collagen. pp. 145-172, Academic Press, Inc., London

Burgeson, R.E. & Nimni, M. Collagen types: molecular structure and tissue distribution. (1992) Clin. Orthop. Rel. Res. **282**, 250-272

Buschmann, M.D., Gluzband, Y.A., Grodzinsky, A.J., Kimura, J.H. & Hunziker, E.B. Chondrocytes in agarose culture synthesise a mechanically functional extracellular matrix. (1992) J. Orthop. Res. **10**, 745-758

Cancedda, R., Descalzi-Cancedda, F. & Castagnola, P. Chondrocyte differentiation. (1995) Int. Rev. Cytol. **159**, 265-358

Castagnola, P., Moro, G., Descalzi-Cancedda, F. & Cancedda, R. Type X collagen synthesis during *in vitro* development of chick embryo tibial chondrocytes. (1986) J. Cell Biol. **102**, 2310-2317

Castagnola, P., Dozin, B., Moro, G. & Cancedda, R. Changes in the expression of collagen genes show two stages in chondrocyte differentiation *in vivo*. (1988) J. Cell Biol. **106**, 461-467

Castagnola, P., Tavella, S., Gerecke, D.R., Dublet, B., Gordon, M.K., Seyer, J., Cancedda, R., van der Rest, M. & Olsen, B.J. Tissue-specific expression of type XIV collagen - a member of the FACIT class of collagens. (1992) Eur. J. Cell Biol. **59**, 340-347

Chan, D., Weng, Y.M., Hocking, A.M., Golub, S., McQuillan, D.J. & Bateman, J.F. Site-directed mutagenesis of human type X collagen. (1996) J. Biol. Chem. **271**, 13566-13572

Chapman, J.A. The regulation of size and form in the assembly of collagen fibrils *in vivo*. (1989) Biopolymers **28**, 1367-1382

Chen, L., Yang, W. & Cole, W.G. Alternative splicing of exon 12 of the COL2A1 gene interrupts the triple helix of type II collagen in the Kneist form of spondyloepiphyseal dysplasia. (1996) J. Orthop. Res. **14**, 712-721

Chen, Q., Gibney, E., Fitch, J.M., Linsenmayer, C.M., Schmid, T. & Linsenmayer, T.F. Long-range movement and fibril association of type X collagen within embryonic cartilage matrix. (1990) Proc. Natl. Acad. Sci. USA **87**, 8046-8050

Cho, M. & Garrant, P.R. Sequential events in the formation of collagen secretion granules with special reference to the development of segment-long-spacing-like aggregates. (1981) Anat. Rec. **199**, 309-320

Choglay, A.A., Purdom, I.F. & Hulmes, D.J.S.H. Procollagen binding to sphingomyelin. (1993) J. Biol. Chem. **268**, 6107-6114

Chu, M. & Prockop, D.J. (1993) in "Connective Tissue and its' Heritable Disorders: Molecular, Genetic and Clinical Aspects" (Royce, P.M. & Steinmann, B. eds.), Collagen: Gene structure. pp. 149-166, Wiley-Liss, Inc., New York

Colige, A., Beschin, A., Samyn, B., Goebels, Y., van Beeumen, J., Nusgens, B.V. & Lapiere, C.M. Characterisation and partial amino acid sequencing of a 107 kDa procollagen I N-proteinase purified by affinity chromatography on immobilized type XIV collagen. (1995) J. Biol. Chem. **270**, 16724-16730

Colige, A., Li, S., Sieron, A.L., Nusgens, B.V., Prockop, D.J. & Lapiere, C.M. cDNA cloning and expression of bovine procollagen I N-proteinase: a new member of the superfamily of zinc-metalloproteinases with binding sites for cells and other matrix components. (1997) Proc. Natl. Acad. Sci. USA **94**, 2374-2379

Cronland, A.L., Smith, B.D. & Kagan, H.M. Binding of lysyl oxidase to fibrils of type I collagen. (1985) Conn. Tiss. Res. **14**, 109-119

Curtin, W.A. & Reville, W.J. Ultrastructural observations of fibril profiles in normal and degenerative human articular cartilage. (1995) Clin. Orthop. Rel. Res. **313**, 224-230

Danielson, K.G., Baribault, H., Holmes, D.F., Graham, H., Kadler, K.E. & Iozzo, R.V. Targeted disruption of decorin leads to abnormal collagen fibril morphology and skin fragility. (1997) J. Cell Biol. **136**, 729-743

Delbruck, A., Dresow, B., Gurr, E., Reale, E. & Schroder, H. *In vitro* culture of human chondrocytes from adult subjects. (1986) Conn. Tiss. Res. **15**, 155-172

Descalzi-Cancedda, F., Gentili, C., Manduca, P. & Cancedda, R. Hypertrophic chondrocytes undergo further differentiation in culture. (1992) J. Cell Biol. **117**, 427-435

Diab, M., Wu, J-J. & Eyre, D.R. Collagen type IX from human cartilage: a structural profile of intermolecular cross-linking sites. (1996) Biochem. J. **314**, 327-332

Dombrowski, K.E. & Prockop, D.J. Cleavage of type I and type II procollagens by type I/II procollagen N-proteinase. (1988) J. Biol. Chem. **263**, 16545-16552

Duance, V.C., Wotton, S.F. & Young, R.D. Type IX collagen function in articular cartilage. (1990) Ann. N. Y. Acad Sci **580**, 480-483

Dublet, B. & van der Rest, M. Type XIV collagen, a new homotrimeric molecule extracted from fetal bovine skin and tendon, with a triple-helical disulfide-bonded domain homologous to type IX and type XII collagens. (1991) J. Biol. Chem. **266**, 6853-6858

Eikenberry, E.F., Mendler, M., Burgin, R., Winterhalter, K.H. & Bruckner, P. (1992) in "Articular Cartilage and Osteoarthritis" (Kuettner, K., Schleyerbach, R., Peyron, J.G. & Hascall, V.C. eds.), Fibrillar organisation in cartilage. pp. 133-149, Raven Press, Ltd., New York

Elima, K. & Vuorio, E. Expression of mRNA's for collagens and other matrix components in dedifferentiating and redifferentiating human chondrocytes in culture. (1989) FEBS Letts **258**, 195-198

Engel, J. & Prockop, D.J. The zipper-like folding of collagen triple helices and the effects of mutations that disrupt the zipper. (1991) Annu. Rev. Biophys. Biophys. Chem. **20**, 137-152

Enobakhare, B.O., Bader, D.L. & Lee, D.A. Quantification of sulphated glycosaminoglycans in chondrocyte/alginate cultures, by use of 1,9-dimethylmethylene blue. (1996) Anal. Biochem. **243**, 189-191

Epstein, E.H. $[\alpha 1(III)]_3$ human skin collagen. (1974) J. Biol. Chem. **249**, 3225-3231

Eyre, D.R., Apon, S., Wu, J-J., Ericsson, L.H. & Walsh, K.A. Collagen type IX: evidence for covalent linkages to type II collagen in cartilage. (1987a) FEBS Letts **220**, 337-341

Eyre, D.R., Wu, J-J. & Apon, S. A growing family of collagens in articular cartilage: identification of 5 genetically distinct types. (1987b) J. Rheumatol. **14**, (suppl 14):25-27

Eyre, D.R., Wu, J-J., Niyibizi, C. & Chun, L.E. (1990) in "Methods in Cartilage Research" (Maroudas, A. & Kuettner, K. eds.), The cartilage collagens: analysis of their cross-linking interactions and matrix organization. pp. 27-58, Academic Press, Ltd., London

Eyre, D.R., Wu, J-J. & Woods, P.E. The cartilage collagens: structural and metabolic studies. (1991a) J. Rheumatol. **18**, (suppl. 27):49-51

Eyre, D.R., Wu, J-J., Woods, P.E. & Weis, M.A. The cartilage collagens and degenerative joint disease. (1991b) Br. J. Rheumatol. **30**, (suppl 1):10-15

Fassler, R., Schnegelsberg, P.N.J., Dausman, J., Shinya, T., Muragaki, Y., McCarthy, M.T., Olsen, B.J. & Jaenisch, R. Mice lacking α1(IX) collagen develop noninflammatory degenerative joint disease. (1994) Proc. Natl. Acad. Sci. USA **91**, 5070-5074

Fernandez-Madrid, F., Noonan, S., Riddle, J., Karvonen, R. & Sasaki, D. Intracellular processing of procollagen induced by the action of colchicine. (1980) J. Anat. **130**, 229-241

Fertala, A., Sieron, S.L., Hojima, Y., Ganguly, A. & Prockop, D.J. Self-assembly into fibrils of collagen II by enzymic cleavage of recombinant procollagen II. (1994) J. Biol. Chem. **269**, 11584-11589

Fife, R.S. & Brandt, K.D. (1993) in "Joint Cartilage Degradation: Basic and Clinical Aspects" (Woessner, J.F. & Howell, D.S. eds.), Extracellular matrix of cartilage: glycoproteins. pp. 139-158, Marcel Dekker, Inc., New York

Fitch, J.M., Linsenmayer, C.M. & Linsenmayer, T.F. Collagen fibril assembly in the developing avian primary corneal stroma. (1994) Invest. Opthalm. Vis. Sci **35**, 862-869

Fleischmajer, R., Timpl, R., Tuderman, L., Raisher, L., Weistner, M., Perlish, J.S. & Graves, P.N. Ultrastructural identification of extension aminopropeptides of type I and type III collagens in human skin. (1981) Proc. Natl. Acad. Sci. USA **78**, 7360-7364

Fleischmajer, R., Perlish, J.S., Timpl, R. & Olsen, B.J. Procollagen intermediates during tendon fibrillogenesis. (1985) J. Histochem. Cytochem. **36**, 1425-1432

Fleischmajer, R., Perlish, J.S., Burgeson, R.E., Shaikh-Bahai, F. & Timpl, R. Type I and type III collagen interactions during fibrillogenesis. (1990) Ann. N. Y. Acad. Sci. **580**, 161-175

Galera, P., Vivien, D., Pronost, S., Bonaventure, J., Redini, F., Loyau, G. & Pujol, J. Transforming growth factor-β1 (TGF-β1) up-regulation of collagen type II in primary cultures of rabbit articular chondrocytes (RAC) involves increased mRNA stability and procollagen processing. (1992) J. Cell. Physiol. **153**, 596-606

Garofalo, S., Metsaranta, M., Ellard, J., Smith, C., Horton, W., Vuorio, E. & de Crombrugghe, B. Assembly of cartilage collagen fibrils is disrupted by overexpression of normal type II collagen in transgenic mice. (1993) Genetics **90**, 3825-3829

Glanville, R.W. (1987) in "Structure and Function of Collagen Types" (Mayne, R. & Burgeson, R.E. eds.), Type IV collagen. pp. 43-80, Academic Press, Inc., London

Gordon, M.K., Fitch, J.M., Foley, J.W., Gerecke, D.R., Linsenmayer, C.M., Birk, D.E. & Linsenmayer, T.F. Type XVII collagen (BP180) in the developing avian cornea. (1997) Invest. Opthalm. Vis. Sci **38**, 153-166

Grandolfo, M., D'Andrea, P., Paoletti, S., Martina, M., Silvestrini, G., Bonucci, E. & Vittur, F. Culture and differentiation of chondrocytes entrapped in alginate gels. (1993) Calcif. Tiss. Int. **52**, 42-48

Gross, J., Highberger, J.H. & Schmitt, F.O. Collagen structures considered as states of aggregation of a kinetic unit. The tropocollagen particle. (1954) Proc. Natl. Acad. Sci. USA **40**, 679-688

Grushko, G., Schneiderman, R. & Maroudas, A. Some biochemical and biophysical parameters for the study of the pathogenesis of osteoarthritis: a comparison between the processes of ageing and degeneration in human hip cartilage. (1989) Conn. Tiss. Res. **19**, 149-176

Guo, J., Jourdian, G.W. & MacCallum, D.K. Culture and growth characteristics of chondrocytes encapsulated in alginate beads. (1989) Conn. Tiss. Res. **19**, 277-297

Hames, B.D. (1990) in "Gel Electrophoresis of Proteins. A Practical Approach" (Hames, B.D. & Rickwood, D. eds.), One-dimensional polyacrylamide gel electrophoresis. pp. 1-147, IRL Press, Oxford

Hardingham, T.E. & Bayliss, M.T. Proteoglycans of articular cartilage: changes in aging and in joint disease. (1990) Sem. Arthr. Rheum. **20**, (suppl 1):12-33

Hardingham, T.E., Venn, G. & Bayliss, M.T. Chondrocyte responses in cartilage and in experimental osteoarthritis. (1991) Br. J. Rheumatol. **30**, (suppl 1):32-37

Hauselmann, H.J., Aydelotte, M.B., Schumacher, B.L., Kuettner, K.E., Gitelis, S.H. & Thonar, E.J.A. Synthesis and turnover of proteoglycans by human and bovine adult articular chondrocytes cultured in alginate beads. (1992) Matrix **12**, 116-129

Hauselmann, H.J., Fernandes, R.J., Mok, S.S., Schmid, T., Block, J.A., Aydelotte, M.B., Kuettner, K.E. & Thonar, E.J.A. Phenotypic stability of bovine articular chondrocytes after long-term culture in alginate beads. (1994) J. Cell Sci. **107**, 17-27

Hauselmann, H.J., Masuda, K., Hunziker, E.B., Neidhart, M., Mok, S.S., Michel, B. & Thonar, E.J.A. Adult human chondrocytes cultured in alginate form a matrix similar to native human articular cartilage. (1996) Am. J. Phsiol. **271**, (cell physiol. 40):C742-C752

Hay, E. (1991) "Cell Biology of Extracellular Matrix". Plenum Press, New York

Hedbom, E. & Heinegard, D. Interaction of a 59 kDa connective tissue protein with collagen I and collagen II. (1989) J. Biol. Chem. **264**, 6898-6905

Hedbom, E. & Heinegard, D. Binding of fibromodulin and decorin to separate sites on fibrillar collagens. (1993) J. Biol. Chem. **268**, 27307-27312

Hojima, Y., McKenzie, J., van der Rest, M. & Prockop, D.J. Type I procollagen N-proteinase from chick embryo tendons. (1989) J. Biol. Chem. **264**, 11336-11345

Hojima, Y., Behta, B., Romanic, A.M. & Prockop, D.J. Cleavage of type I procollagen by C- and N-proteinases is more rapid if the substrate is aggregated with dextran sulphate or polyethylene glycol. (1994a) Anal. Biochem. **223**, 173-180

Hojima, Y., Morgelin, M.M., Engel, J., Boutillon, M., van der Rest, M., McKenzie, J., Chen, G., Rafi, N., Romanic, A.M. & Prockop, D.J. Characterisation of type I procollagen N-proteinase from fetal bovine tendon and skin. (1994b) J. Biol. Chem. **269**, 11381-11390

Holmes, D.F., Capaldi, M.J. & Chapman, J.A. Reconstitution of collagen fibrils *in vitro*; the assembly process depends on the initiation procedure. (1986) Int. J. Biol. Macromol. **8**, 161-166

Holtzer, H., Abbott, J., Lash, J. & Holtzer, S. The loss of phenotypic traits by differentiated cells *in vitro*. I. Dedifferentiation of cartilage cells. (1960) Proc. Natl. Acad. Sci. USA **46**, 1533-1542

Hopkinson, S.B. & Jones, J.C.R. Type XVII collagen and collagen-like molecules: related by more than a common motif. (1996) Sem. Cell Devel. Biol. **7**, 659-666

Horton, W. (1993) in "Connective Tissue and it's Heritable Disorders: Molecular, Genetic and Medical Aspects" (Royce, P.M. & Steinmann, B. eds.), Morphology of connective tissue: cartilage. pp. 73-84, Wiley-Liss, Inc., New York

Horton, W. & Hecht, J.T. (1993) in "Connective Tissue and it's Heritable Disorders: Molecular, Genetic and Medical Aspects" (Royce, P.M. & Steinmann, B. eds.), The chondrodysplasias. pp. 641-675, Wiley-Liss, Inc., New York

Horwitz, A.F. Integrins and health. (1997) Scientific American **276**, 46-53

Horwitz, A.L. & Dorfman, A. The growth of cartilage cells in soft agar and liquid suspension. (1970) J. Cell Biol. **45**, 434-438

Huber, S., van der Rest, M., Bruckner, P., Rodriguez, E., Winterhalter, K.H. & Vaughan, L. Identification of the type IX collagen polypeptide chains - the alpha-2(IX) polypeptide carries the chondroitin sulfate chain(s). (1986) J. Biol. Chem. **261**, 5965-5968

Huber, S., Winterhalter, K.H. & Vaughan, J. Isolation and sequence analysis of the glycosaminoglycan attachment site of type IX collagen. (1988) J. Biol. Chem. **263**, 752-756

Huebner, K., Cannizzario, L.A., Jabs, E.W., Kivirikko, S., Manzone, H., Pihlajaniemi, T. & Myers, J.C. Chromosomal assignment of a gene encoding a new collagen type (COL15A1) to 9q21 - q22. (1992) Genomics **14**, 220-224

Hulmes, D.J.S., Bruns, R.R. & Gross, J. On the state of aggregation of newly secreted procollagen. (1983) Proc. Natl. Acad. Sci. USA **80**, 388-392

Hulmes, D.J.S., Kadler, K.E., Mould, A.P., Hojima, Y., Holmes, D.F., Cummings, C., Chapman, J.A. & Prockop, D.J. Pleomorphism in type I collagen fibrils produced by persistance of the procollagen N-propeptide. (1989) J. Mol. Biol. **210**, 337-345

Hulmes, D.J.S. The collagen superfamily - diverse structures and assemblies. (1992) Ess. Biochem. **27**, 49-67

Inoguchi, K., Yoshioka, H., Khaleduzzaman, M. & Ninomiya, Y. The mRNA for α1(XIX) collagen chain, a new member of FACITs, contains a long unusual 3' untranslated region and displays many unique splicing variants. (1995) J. Biochem. **117**, 137-146

Iozzo, R.V. & Murdoch, A.D. Proteoglycans of the extracellular matrix environment: clues from the gene and protein side offer novel perspectives in molecular diversity and function. (1996) FASEB J. **10**, 598-614

Kadler, K.E. Extracellular Matrix .1. Fibril-forming collagens. (1994) Protein Profile **1**, 491-638

Kadler, K.E., Lightfoot, S.J. & Watson, R.B. Procollagen N-peptidases: procollagen N-proteinases. (1995) Meth. Enzymol. **248**, 756-771

Kadler, K.E. & Watson, R.B. Procollagen C-peptidase: procollagen C-proteinase. (1995) Meth. Enzymol. **248**, 771-781

Kadler, K.E., Hulmes, D.J.S., Hojima, Y. & Prockop, D.J. Assembly of type I collagen fibrils *de novo* by the specific enzymic cleavage of pC collagen. (1990) Ann. N. Y. Acad. Sci. **580**, 214-224

Kadler, K.E., Holmes, D.F., Trotter, J.A. & Chapman, J.A. Collagen fibril formation. (1996) Biochem. J. **316**, 1-11

Kagan, H.M. (1986) in "Regulation of Matrix Accumulation: Biology of the Extracellular Matrix" (Mecham, R.P. ed.), Characterisation and regulation of lysyl oxidase. pp. 321-398, Academic Press, Orlando

Kato, S., Spinale, F.G., Tanaka, R., Johnson, W., Cooper, G. & Zile, M.R. Inhibition of collagen cross-linking: effects on fibrillar collagen and ventricular diastolic function. (1995) Am. J. Physiol. **269**, H863-H868

Kaufman, S.F., Ewing, C.M. & Shaper, J.H. The erasable western blot. (1987) Anal. Biochem. **161**, 89-95

Keene, D.R., Lunstrum, G.P., Morris, N.P., Stoddard, D.W. & Burgeson, R.E. Two type XII-like collagens localize to the surface of banded collagen fibrils. (1991) J. Cell Biol. **113**, 971-978

Keene, D.R., Thom-Oxford, J. & Morris, N.P. Ultrastructural localisation of collagen types II, IX and XI in the growth plate of human rib and fetal bovine epiphyseal cartilage: type XI collagen is restricted to thin fibrils. (1995) J. Histochem. Cytochem. **43**, 967-979

Kefalides, N.A. Isolation of a collagen from basement membranes containing three identical chains. (1971) Biochem. Biophys. Res. Comm. **45**, 226-234

Kessler, E., Takahara, K., Biniaminov, L., Brusel, M. & Greenspan, D.S. Bone morphogenetic protein-1 - the type I procollagen C-proteinase. (1996) Science **271**, 360-362

Kielty, C.M., Hulmes, D.J.S., Schor, S.L. & Grant, M.E. Embryonic chicken cartilage collagens: Differences in the low-M_r species present in sternal cartilage and tibiotarsal articular cartilage. (1984) FEBS Letts **169**, 179-184

Kielty, C.M., Hopkinson, I. & Grant, M.E. (1993) in "Connective Tissue and it's Heritable Disorders: Molecular, Genetic and Medical Aspects" (Royce, P.M. & Steinmann, B. eds.), Collagen: The collagen family: Structure, assembly, and organisation in the extracellular matrix. pp. 103-147, Wiley-Liss, Inc., New York

Kimura, J.H. & Kuettner, K.E. (1986) in "Articular Cartilage Biochemistry" (Kuettner, K., Schleyerbach, R. & Hascall, V.C. eds.), Studies on the synthesis and assembly of cartilage proteoglycan. pp. 113-124, Raven Press, New York

Kleman, J., Hartmann, D.J., Ramirez, F. & van der Rest, M. The human rhabdomyosarcoma cell line A204 lays down a highly soluble matrix composed mainly of α1 type XI and α2 type V collagen chains. (1992) Eur. J. Biochem. **210**, 329-335

Kohn, L.D., Isersky, C., Zupnik, J., Lenaers, A., Lee, G. & Lapiere, C.M. Calf tendon procollagen peptidase: its purification and endopeptidase mode of action. (1974) Proc. Natl. Acad. Sci. USA **71**, 40-44

Koyabashi, K., Ito, T. & Hoshino, T. Electron microscopic demonstration of acid-labile, 4D-staggered intermolecular association of collagen formed *in vitro*. (1985) Collagen Rel. Res. **5**, 253-260

Koyano, Y., Hejna, M., Flechtenmacher, J., Schmid, T., Thonar, E.J.A. & Mollenhauer, J. Collagen and proteoglycan production by bovine fetal and adult chondrocytes under low levels of calcium and zinc ions. (1996) Conn. Tiss. Res. **34**, 213-225

Kuettner, K.E., Thonar, E.J.A. & Aydelotte, M.B. (1990) in "Mechanisms of Articular Cartilage Damage and Repair in Osteoarthritis" (Muir, H., Hirohata, K. & Shichikawa, K. eds.), Articular cartilage - structure and chondrocyte metabolism. pp. 11-30, Hogrefe and Huber Publishers, Toronto

Kuettner, K.E., Aydelotte, M.B. & Thonar, E.J.A. Articular cartilage matrix and structure: a minireview. (1991) J. Rheumatol. **18**, (suppl 27):46-48

Kuhn, K. (1987) in "Structure and Function of Collagen Types" (Mayne, R. & Burgeson, R.E. eds.), The classical collagens: types I, II and III. pp. 1-42, Academic Press, Inc., Orlando, Florida

Kwan, A.P.L., Cummings, C., Chapman, J.A. & Grant, M.E. Macromolecular organisation of chicken type X collagen *in vitro*. (1991) J. Cell Biol. **114**, 597-604

Laemmli, U.K. Cleavage of structural proteins during the assembly of the head of bacteriophage. (1970) Nature **227**, 680-685

Lapiere, C.M., Lenaers, A. & Kohn, L.D. Procollagen peptidase: an enzyme excising the coordination peptides of procollagen. (1971) Proc. Natl. Acad. Sci. USA **68**, 3054-3058

Lee, G.M., Poole, C.A., Kelley, S.S., Chang, J. & Caterson, B. Isolated chondrons: a viable alternative for studies of chondrocyte metabolism *in vitro*. (1997) Osteoarth. Cart. **5**, 261-274

Lee, S.L. & Piez, K.A. Type II collagen from lathyritic rat chondrosarcoma: preparation and *in vitro* fibril formation. (1983) Collagen Rel. Res **3**, 89-103

Lefkoe, T.P., Nalin, A.M., Clark, J.M., Reife, R.A., Sugai, J. & Sandell, L.J. Gene expression of collagen types IIA and IX correlates with ultrastructural events in early osteoarthrosis: new applications of the rabbit meniscectomy model. (1997) J. Rheumatol. **24**, 1155-1163

Levene, C.I. & Gross, J. Alterations in state of molecular aggregation of collagen induced in chick embryos by β-aminopropionitrile (lathyrus factor). (1959) J. Exp. Med. **110**, 771-790

Li, K., Tamai, K. & Uitto, J. Cloning of type XVII collagen. (1993) J. Biol. Chem. **268**, 8825-8834

Li, S., Prockop, D.J., Helminen, H.J., Fassler, R., Lapvetelainen, T., Kiraly, K., Peltarri, A., Arokoski, J., Lui, H., Arita, M. & Khillan, J.S. Transgenic mice with targeted inactivation of the COL2A1 gene for collagen II develop a skeleton with membranous and periosteal bone but no endochondral bone. (1995) Genes Develop. **9**, 2821-2830

Li, Y., Lacerda, D.A., Warman, M.L., Beier, D.R., Yoshioka, H., Ninomiya, Y., Thom-Oxford, J., Morris, N.P., Andrikopoulos, K., Ramirez, F., Wardell, B.B., Lifferth, G.D., Teuscher, C., Woodward, S.R., Taylor, B.A., Seegmiller, R. & Olsen, B.J. A fibrillar collagen gene, COL11A1, is essential for skeletal morphogenesis. (1995) Cell **80**, 423-430

Linsenmayer, T.F., Gibney, E., Igoe, F., Gordon, M.K., Fitch, J.M., Fessler, L.I. & Birk, D.E. Type V collagen: molecular structure and fibrillar organization of the chicken α1(V) NH_2-terminal domain, a putative regulator of corneal fibrillogenesis. (1993) J. Cell Biol. **121**, 1181-1189

Loeser, R.F. Integrin-mediated attachment of articular chondrocytes to extracellular matrix proteins. (1993) Arth. Rheum. **36**, 1103-1110

MacBeath, J.R.E., Shackleton, D.R. & Hulmes, D.J.S. Tyrosine-rich acidic matrix protein (TRAMP) accelerates collagen fibril formation *in vitro*. (1993) J. Biol. Chem. **268**, 19826-19832

Mallein-Gerin, F. & van der Rest, M. La culture de chondrocytes: outil d'analyse de la differentiation et de l'organisation moleculaire du cartilage. (1996) Medecine **12**, 1087-1096

Marchant, J.K., Hahn, R.A., Linsenmayer, T.F. & Birk, D.E. Reduction of type V collagen using a dominant-negative strategy alters the regulation of fibrillogenesis and results in the loss of corneal-specific fibril morphology. (1996) J. Cell Biol. **135**, 1415-1426

Maroudas, A., Katz, E.P., Wachtel, E.J., Mizrahi, J. & Soudry, M. (1986) in "Articular Cartilage Biochemistry" (Kuettner, K., Schleyerbach, R. & Hascall, V.C. eds.), Physiochemical properties and functional behaviour of normal and osteoarthritic human cartilage. pp. 311-330, Raven Press, New York

Matyas, J.R., Adams, M.E., Huang, D. & Sandell, L.J. Major role of collagen IIB in the elevation of total type II procollagen messenger RNA in the hypertrophic phase of experimental osteoarthritis. (1997) Arth. Rheum. **40**, 1046-1049

Mayne, R. Cartilage collagens: What is their function and are they involved in articular disease? (1989) Arth. Rheum. **32**, 241-246

Mayne, R. & Brewton, R.G. New members of the collagen superfamily. (1993) Curr. Opin. Cell Biol. **5**, 883-890

Mayne, R., Brewton, R.G., Mayne, P.M. & Baker, J.R. Isolation and characterisation of type V/type XI collagen present in bovine vitreous. (1993) J. Biol. Chem. **268**, 9381-9386

McCormick, D., van der Rest, M., Goodship, J., Lozano, G., Ninomiya, Y. & Olsen, B.J. Structure of the glycosaminoglycan domain in the type IX collagen-proteoglycan. (1987) Proc. Natl. Acad. Sci. USA **84**, 4044-4048

McPherson, J.M., Wallace, D.G., Sawamura, S.J., Conti, A., Condell, R.A., Wade, S. & Piez, K.A. Collagen fibrillogenesis *in vitro*: a characteristic of fibril quality as a function of assembly conditions. (1985) Collagen Rel. Res **5**, 119-135

Mendler, M., Eich-Bender, S.G., Vaughan, L., Winterhalter, K.H. & Bruckner, P. Cartilage contains mixed fibrils of collagen types II, IX and XI. (1989) J. Cell Biol. **108**, 191-197

Merker, H., Lilja, S., Barrach, H.J. & Gunter, T. Formation of an atypical collagen and cartilage pattern in limb bud cultures by highly sulfated GAG. (1978) Virchows Arch. A Path. Anat. Histol. **380**, 11-30

Miller, E.J. & Matukas, V.J. Chick cartilage collagen: a new type of α1chain not present in bone or skin of the species. (1969) Proc. Natl. Acad. Sci. USA **64**, 1264-1268

Miller, E.J. & Gay, S. The collagens: an overview and update. (1987) Meth. Enzymol. **144**, 3-40

Miyahara, M., Hayashi, K., Berger, J., Tanzawa, K., Njieha, F.K., Trelstad, R.L. & Prockop, D.J. Formation of collagen fibrils by enzymic cleavage of precursors of type I collagen *in vitro*. (1984) J. Biol. Chem. **259**, 9891-9898

Mizuno, K. & Hayashi, T. Peculiar effect of urea on the interaction of type I collagen with heparin on chromatography. (1994) J. Biochem. **116**, 1257-1263

Mizuno, K., Adachi, E. & Hayashi, T. Reconstitution of banding fibrils from the subtypes of type V collagen with the chain compositions of $[\alpha1(V)]_2\alpha2(V)$ and $\alpha1(V)\alpha2(V)\alpha3(V)$. (1997) Matrix Biol. **16**, 76

Mok, S.S., Masuda, K., Hauselmann, H.J., Aydelotte, M.B. & Thonar, E.J.A. Aggrecan synthesised by mature bovine chondrocytes suspended in alginate. (1994) J. Biol. Chem. **269**, 33021-33027

Morikawa, T., Tuderman, L. & Prockop, D.J. Inhibitors of procollagen N-protease. Synthetic peptides with sequences similar to the cleavage site in the pro1α(I) chain. (1980) Biochem. **19**, 2646-2650

Morris, N.P. & Bachinger, H.P. Type XI collagen is a heterotrimer with the composition (1α2α3α) retaining nontriple-helical domains. (1987) J. Biol. Chem. **262**, 11345-11350

Morris, N.P., Watt, S.L., Davis, J.M. & Bachinger, H.P. Unfolding intermediates in the triple helix to coil transitions of bovine type XI collagen and human type V collagens $\alpha 1_2\alpha 2$ and α1α2α3. (1990) J. Biol. Chem. **265**, 10081-10087

Morrissey, J.H. Silver stain for proteins in polyacrylamide gels: a modified procedure with enhanced uniform sensitivity. (1981) Anal. Biochem. **117**, 307-310

Mould, A.P., Hulmes, D.J.S., Holmes, D.F., Cummings, C., Sear, C.H.J. & Chapman, J.A. D-periodic assemblies of type I procollagen. (1990) J. Mol. Biol. **211**, 581-594

Muir, H.I. (1990) in "Mechanisms of Articular Damage and Repair in Osteoarthritis" (Muir, H., Hirohata, K. & Shichikawa, K. eds.), Biochemical basis for cartilage degeneration, destruction and loss of function in osteoarthritis. pp. 31-42, Hogrefe and Huber Publishers, Toronto

Muir, H.I. The chondrocyte, architect of cartilage. (1995) Bioess. **17**, 1039-1048

Muller-Glauser, W., Humbel, B., Glatt, M., Strauli, P., Winterhalter, K.H. & Bruckner, P. On the role of type IX collagen in the extracellular matrix of cartilage: type IX collagen is localized to intersections of collagens fibrils. (1986) J. Cell Biol. **102**, 1931-1939

Nah, H., Barembaum, M. & Upholt, W.B. The chicken α1(XI) collagen gene is widely expressed in embryonic tissues. (1992) J. Biol. Chem. **267**, 22581-22586

Nakata, K., Ono, K., Miyazaki, J., Olsen, B.J., Muragaki, Y., Adachi, E., Yamamura, K. & Kimura, J.H. Osteoarthritis associated with mild chondrodysplasia in transgenic mice expressing α1(IX) collagen chains with a central deletion. (1993) Proc. Natl. Acad. Sci. USA **90**, 2870-2874

Neame, P.J., Young, C.N. & Treep, J.T. Isolation and primary structure of PARP, a 24 kDa proline- and arginine-rich protein from bovine cartilage closely related to the NH_2-terminal domain of collagen α1(XI). (1990) J. Biol. Chem. **265**, 20401-20408

Neame, P.J. (1993) in "Joint Cartilage Degradation: Basic and Clinical Aspects" (Woessner, J.F. & Howell, D.S. eds.), Extracellular Matrix of Cartilage: Proteoglycans. pp. 109-138, Marcel Dekker, Inc., New York

Nishimura, I., Muragaki, Y. & Olsen, B.J. Tissue-specific forms of type IX collagen-proteoglycan arise from the use of two widely separated promoters. (1989) J. Biol. Chem. **264**, (33):20033-20041

Niyibizi, C. & Eyre, D.R. Identification of the cartilage α1(XI) chain in type V collagen from bovine bone. (1989) FEBS Letts **242**, 314-318

O'Keefe, R.J., Puzas, J.E., Loveys, L., Hicks, D.G. & Rosier, R.N. Analysis of type II and type X collagen synthesis in cultured growth plate chondrocytes by *in situ* hybridization: rapid induction of type X collagen in culture. (1994) J. Bone Min. Res. **9**, 1713-1722

Oldberg, A. A collagen-binding 59-kd protein (fibromodulin) is structurally related to the small interstitial proteoglycans PG-S1 and PG-S2 (decorin). (1989) EMBO J. **8**, 2601-2604

Olsen, B.J. Mutations in collagen genes resulting in metaphyseal and epiphyseal dysplasias. (1995) Bone **17**, 45S-49S

Olsen, B.J. Collagen IX. (1997) Int. J. Biochem. Cell Biol. **29**, 555-558

Pan, T., Zhang, R., Mattei, M., Timpl, R. & Chu, M. Cloning and chromosomal location of human α1(XVI) collagen. (1992) Proc. Natl. Acad. Sci. USA **89**, 6565-6569

Perez-Tamayo, R. The occurrence and significance of SLS crystallites *in vivo*. (1972) Conn. Tiss. Res. **1**, 55-60

Pesciotta, D.M., Curran, S. & Olsen, B.J. (1982) in "Immunochemistry of the Extracellular Matrix" (Furthmayr, H. ed.), Preparation of antisera specific for the amino and carboxyl propeptides of type I and type II procollagens. pp. 91-109, CRC Press, Boca Raton

Petit, B., Ronziere, M.C., Hartmann, D.J. & Herbage, D. Ultrastructural organisation of type XI collagen in fetal bovine epiphyseal cartilage. (1993) Histochem. **100**, 231-239

Petit, B., Masuda, K., D'Souza, A.L., Otten, L., Pietryla, D., Hartmann, D.J., Morris, N.P., Uebelhart, D., Schmid, T. & Thonar, E.J.A. Characterisation of cross-linked collagens synthesized by mature articular chondrocytes cultured in alginate beads: comparison of two distinct matrix compartments. (1996) Exp. Cell Res. **225**, 151-161

Pihlajaniemi, T. & Tamminen, M. The α1 chain of type XIII collagen consists of three collagenous and four noncollagenous domains and its primary transcript undergoes complex alternative splicing. (1990) J. Biol. Chem. **265**, 16922-16928

Poole, A.R., Pidoux, I., Reiner, A., Rosenburg, L.C., Hollister, D.W., Murray, L.W. & Rimoin, D. Kneist dysplasia is characterised by an apparent abnormal processing of the C-propeptide of type II cartilage collagen resulting in imperfect fibril assembly. (1988) J. Clin. Invest. **81**, 579-589

Poole, A.R., Rosenburg, L.C., Reiner, A., Ionescu, M., Bogoch, E. & Roughley, P.J. Contents and distributions of the proteoglycans decorin and biglycan in normal and osteoarthritic human articular cartilage. (1996) J. Orthop. Res. **14**, 681-689

Poole, C.A., Flint, M.H. & Beaumont, B.W. Morphology of the pericellular capsule in articular cartilage revealed by hyaluronidase digestion. (1985) J. Ultrastruc. Res. **91**, 13-23

Poole, C.A., Wotton, S.F. & Duance, V.C. Localisation of type IX collagen in chondrons isolated from porcine articular cartilage and rat chondrosarcoma. (1988) Histochem. J. **20**, 567-574

Poole, C.A. (1993) in "Joint Cartilage Degradation: Basic and Clinical Aspects" (Woessner, J.F. & Howell, D.S. eds.), Structure and function of cartilage. pp. 1-36, Marcel Dekker, Inc., New York

Prockop, D.J. & Hulmes, D.J.S. (1994) in "Extracellular Matrix Assembly and Structure" (Yurchenko, P.D., Birk, D.E. & Mecham, R.P. eds.), Assembly of collagen fibrils *de novo* from soluble precursors: polymerisation and copolymerisation of procollagen, pN-collagen and mutated collagens. pp. 47-90, Academic Press, Inc., London

Prockop, D.J. & Kivirikko, K.I. Collagens: Molecular biology, diseases and potentials for therapy. (1995) Ann. Rev. Biochem. **64**, 403-434

Ramdi, H., Legay, C. & Lievremont, M. Influence of matricial molecules on growth and differentiation of entrapped chondrocytes. (1993) Exp. Cell Res. **207**, 449-454

Redini, F., Min, W., Demoor-Fossard, M., Boittin, M. & Pujol, J. Differential expression of membrane-anchored proteoglycans in rabbit articular chondrocytes cultured in monolayers and in alginate beads: effect of transforming growth factor-β1. (1997) Biochim. Biophys. Acta **1355**, 20-32

Reese, C.A. & Mayne, R. Minor collagens of chicken hyaline cartilage. (1981) Biochem. **20**, 5443-5448

Reese, C.A., Weidemann, H., Kuhn, K. & Mayne, R. Characterisation of a highly soluble collagenous molecule isolated from chicken hyaline cartilage. (1982) Biochem. **21**, 826-830

Rehn, M. & Pihlajaniemi, T. Type XV and XVIII collagens, a subgroup within the family of collagens. (1996) Sem. Cell Devel. Biol. **7**, 673-679

Rhodes, K. & Miller, E.J. Physiochemical characterisation and molecular organisation of the collagen A and B chains. (1978) Biochem. **17**, 3442-3448

Roughley, P.J. & Lee, E.R. Cartilage proteoglycans: structure and potential functions. (1994) Microsc. Res. Tech. **28**, 385-397

Rousseau, J., Farjanel, J., Boutillon, M., Hartmann, D.J., van der Rest, M. & Moradi-Ameli, M. Processing of type XI collagen: determination of the matrix forms of α1(XI) chain. (1996) J. Biol. Chem. **271**, 23743-23748

Ryan, M.C. & Sandell, L.J. Differential expression of a cysteine-rich domain in the amino-terminal propeptide of type II (cartilage) procollagen by alternative splicing of mRNA. (1990) J. Biol. Chem. **265**, 10334-10339

Saarela, J., Rehn, M., Ylikarppa, R., Autioharmainen, H. & Pihlajaniemi, T. Tissue distribution of type XVIII collagen. (1996) Mol. Biol. Cell **7**, Abs. 3363

Sandell, L.J., Morris, N.P., Robbins, J.R. & Goldring, M.B. Alternatively spliced type II procollagen mRNAs define distinct populations of cells during vertebral development: differential expression of the amino-propeptide. (1991) J. Cell Biol. **114**, 1307-1319

Sandy, J.D., Adams, M.E., Billingham, M.E.J., Plaas, A. & Muir, H.I. *In vivo* and *in vitro* stimulation of chondrocyte biosynthetic activity in early experimental osteoarthritis. (1984) Arth. Rheum. **27**, 388-397

Schenk, R.K., Eggli, P.S. & Hunziker, E.B. (1986) in "Articular Cartilage Biochemistry" (Kuettner, K., Schleyerbach, R. & Hascall, V.C. eds.), Articular cartilage morphology. pp. 3-22, Raven Press, New York

Schmid, T. & Linsenmayer, T.F. Immunoelectron microscopy of type X collagen: supramolecular forms within embryonic chick cartilage. (1990) Develop. Biol. **138**, 53-62

Seegmiller, R., Fraser, F.C. & Sheldon, H. A new chondrodystrophic mutant in mice. (1971) J. Cell Biol. **48**, 580-593

Seegmiller, R., Ferguson, C.C. & Sheldon, H. Studies on cartilage. IV. A genetically determined defect in tracheal cartilage. (1972) J. Ultrastruc. Res. **38**, 288-301

Shaw, L.M. & Olsen, B.J. FACIT collagens: diverse molecular bridges in extracellular matrices. (1991) TIBS **16**, 191-194

Shimokomaki, M., Wright, D.W., Irwin, M.I., van der Rest, M. & Mayne, R. The structure and macromolecular organization of type IX collagen in cartilage. (1990) Ann. N. Y. Acad Sci **580**, 1-7

Silver, D., Miller, J., Harrison, R. & Prockop, D.J. Helical model of nucleation and propagation to account for the growth of type I collagen fibrils from symmetrical pointed tips: a special example of self-assembly of rod-like monomers. (1992) Proc. Natl. Acad. Sci. USA **89**, 9860-9864

Smith, G.N., Williams, J.M. & Brandt, K.D. Effect of polyanions on fibrillogenesis by type XI collagen. (1987) Collagen Rel. Res **7**, 17-25

Smith, G.N. & Brandt, K.D. Hypothesis: can type IX collagen "glue" together intersecting type II fibers in articular cartilage matrix? A proposed mechanism. (1992) J. Rheumatol. **19**, 14-17

Steinmann, B., Royce, P.M. & Superti-Furga, A. (1993) in "Connective Tissue and its Heritable Disorders: Molecular, Genetic and Medical Aspects (Royce, P.M. & Steinmann, B. eds.), The Ehlers-Danlos syndrome. pp. 351-408, Wiley-Liss, Inc., New York

Stockwell, R.A. (1979) "Biology of Cartilage Cells". Cambridge University Press, Cambridge

Studer, D., Chiquet, M. & Hunziker, E.B. Evidence for a distinct water-rich layer surrounding collagen fibrils in articular cartilage extracellular matrix. (1996) J. Struct. Biol. **117**, 81-85

Svoboda, K.K., Nishimura, I., Sugrue, S.P., Ninomiya, Y. & Olsen, B.J. Embryonic chicken cornea and cartilage synthesize type IX collagen molecules with different amino-terminal domains. (1988) Proc. Natl. Acad. Sci. USA **85**, 7496-7500

Tang, S.S., Trackman, P.C. & Kagan, H.M. Reaction of aortic lysyl oxidase with beta-aminopropionitrile. (1983) J. Biol. Chem. **258**, 4331-4338

Tanzawa, K., Berger, J. & Prockop, D.J. Type I procollagen N-proteinase from whole chick embryos. (1985) J. Biol. Chem. **260**, 1120-1126

Thom, J.R. & Morris, N.P. Biosynthesis and proteolytic processing of type XI collagen in embryonic chick sterna. (1991) J. Biol. Chem. **266**, 7262-7269

Thom-Oxford, J., Doege, K.J. & Morris, N.P. Alternative exon splicing within the amino-terminal nontriple-helical domain of the rat pro-α1(XI) collagen chain generates multiple forms of the mRNA transcript which exhibit tissue-dependent variation. (1995) J. Biol. Chem. **270**, 9478-9485

Thompson, A.Y., Piez, K.A. & Seyedin, S.M. Chondrogenesis in agarose gel culture. (1985) Exp. Cell Res. **157**, 483-494

Timpl, R. & Engel, J. (1987) in "Structure and Function of Collagen Types" (Mayne, R. & Burgeson, R.E. eds.), Type VI collagen. pp. 105-143, Academic Press, Inc., London

Towbin, H., Staehelin, T. & Gordon, J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedures and applications. (1979) Proc. Natl. Acad. Sci. USA **76**, 4350-4354

Tschan, T., Hoerler, I., Houze, Y., Winterhalter, K.H., Richter, C. & Bruckner, P. Resting chondrocytes in culture survive without growth factors, but are sensitive to toxic oxygen metabolites. (1990) J. Cell Biol. **111**, 257-260

Tschan, T., Bohme, K., Conscience-Egli, M., Zenke, G., Winterhalter, K.H. & Bruckner, P. Autocrine or paracrine transforming growth factor-β modulates the phenotype of chick embryo sternal chondrocytes in serum-free agarose culture. (1993) J. Biol. Chem. **268**, 5156-5161

Tuderman, L., Kivirikko, K.I. & Prockop, D.J. Partial purification and characterisation of a neutral protease which cleaves the N-terminal propeptides from procollagen. (1978) Biochem. **17**, 2948-2954

Tuderman, L. & Prockop, D.J. Procollagen N-proteinase: properties of the enzyme purified from chick embryo tendons. (1982) Eur. J. Biochem. **125**, 545-549

Urban, J.P.G. The chondrocyte: A cell under pressure. (1994) Br. J. Rheumatol. **33**, 901-908

van der Rest, M., Mayne, R., Ninomiya, Y., Seidah, N.G., Chretien, M. & Olsen, B.J. The structure of type IX collagen. (1985) J. Biol. Chem. **260**, 220-225

van der Rest, M. & Mayne, R. (1987) in "Structure and Function of Collagen Types" (Mayne, R. & Burgeson, R.E. eds.), Type IX collagen. pp. 195-221, Academic Press, Inc., London

van der Rest, M. & Mayne, R. Type IX collagen proteoglycan from cartilage is covalently cross-linked to type II collagen. (1988) J. Biol. Chem. **263**, 1615-1618

van der Rest, M. & Garronne, R. Collagen family of proteins. (1991) FASEB J. **5**, 2814-2823

van der Rest, M., Garronne, R. & Herbage, D. Collagen: a family of proteins with many facets. (1993) Adv. Mol. Cell Biol. **6**, 1-67

van der Rest, M. & Dublet, B. Type XII and type XIV collagens: interfibrillar constituents of dense connective tissues. (1996) Sem. Cell Devel. Biol. **7**, 639-648

Vasios, G., Nishimura, I., Konomi, H., van der Rest, M., Ninomiya, Y. & Olsen, B.J. Cartilage type IX collagen-proteoglycan contains a large amino-terminal globular domain encoded by multiple exons. (1988) J. Biol. Chem. **263**, 2324-2329

Vaughan, L., Huber, S., Chiquet, M. & Winterhalter, K.H. A major six-armed glycoprotein from embryonic cartilage. (1987) EMBO J. **6**, 349-353

Vaughan, L., Mendler, M., Huber, S., Bruckner, P., Winterhalter, K.H., Irwin, M.I. & Mayne, R. D-periodic distribution of collagen type IX along cartilage fibrils. (1988) J. Cell Biol. **106**, 991-997

Vogel, K.G., Paulsson, M. & Heinegard, D. Specific inhibition of type I and type II collagen fibrillogenesis by the small proteoglycan of tendon. (1984) Biochem. J. **223**, 587-597

Von der Mark, K. & Mollenhauer, J. Annexin V interactions with collagen. (1997) Cell Mol. Life Sci. **53**, 539-545

Vornehm, S.I., Dudhia, J., von der Mark, K. & Aigner, T. Expression of collagen types IX and XI and other major cartilage matrix components by human fetal chondrocytes *in vivo*. (1996) Matrix Biol. **15**, 91-98

Wallis, G.A. The importance of being sulphated. (1995) Curr. Biol. **5**, 225-227

Wallis, G.A. Bone growth: coordinating chondrocyte differentiation. (1996) Curr. Biol. **12**, 1577-1580

Wardale, R.J. & Duance, V.C. Quantification and immunolocalisation of porcine articular and growth plate cartilage collagens. (1993) J. Cell Sci. **105**, 975-984

Warshawsky, H. The presence of atypical collagen fibrils in EDTA decalcified predentine and dentine of rat incisors. (1972) Arch. Oral Biol. 1745-1754

Weinstock, M. & Leblond, C.P. Synthesis, migration, and release of precursor collagen by odontoblasts as visualized by autoradiography after [^{3}H]proline administration. (1974) J. Biol. Chem. **60**, 92-127

Weinstock, M. Centrosymmetrical cross-banded structures in the matrix of rat incisor predentin and dentin. (1977) J. Ultrastruc. Res. **61**, 218-229

Weistner, M., Krieg, T., Horlein, D., Glanville, R.W., Fietzek, P. & Muller, P.K. Inhibiting effect of procollagen peptides on collagen biosynthesis in fibroblast cultures. (1979) J. Biol. Chem. **254**, 7016-7023

Wight, T.N., Heinegard, D. & Hascall, V. (1991) in "Cell Biology of Extracellular Matrix" (Hay, E.D. ed.), Proteoglycans: Structure and Function. pp. 45-78, Plenum Press, New York

Williams, B.R., Gelman, R.A., Poppke, D.C. & Piez, K.A. Collagen fibril formation (Optimal *in vitro* conditions and preliminary kinetic results). (1978) J. Biol. Chem. **235**, 6578-6585

Winterbottom, N., Tondravi, M.M., Harrington, T.L., Klier, F.G., Vertel, B.M. & Goetink, P.F. Cartilage matrix protein is a component of the collagen fibril of cartilage. (1992) Develop. Dynam. **193**, 266-276

Winterpacht, A., Hilbert, M., Schwarze, U., Mundlos, S., Spranger, J. & Zabel, B. Kneist and Stickler dysplasia phenotypes caused by collagen type II gene (COL2A1) defect. (1993) Nature Genet. **3**, 323-326

Winterpacht, A. Alternative splicing as the result of a type II procollagen gene (COL2A1) mutation in a patient with Kneist dysplasia. (1994) Hum. Mol. Genet. **3**, 1891-1893

Winterpacht, A., Superti-Furga, A., Schwarze, U., Stoss, H., Steinmann, B., Spranger, J. & Zabel, B. The deletion of six amino acids at the C-terminus of the α1(II) chain causes overmodification of type II and type XI collagen: further evidence for the association between small deletions in COL2A1 and Kneist dysplasia. (1996) J. Med. Genet. **33**, 649-654

Woessner, J.F. The determination of hydroxyproline in tissue and protein samples containing small proportions of this imino acid. (1961) Arch. Biochem. Biophys. **93**, 440-447

Wood, G.C. The formation of fibrils from collagen solutions .2. A mechanism of collagen fibril formation. (1960) Biochem. J. **75**, 598-605

Wood, G.C. & Keech, M.K. The formation of fibrils from collagen solutions .1. The effect of experimental conditions: kinetic and electron microscope studies. (1960) Biochem. J. **75**, 588-598

Wotton, S.F., Duance, V.C. & Fryer, P.R. Type IX collagen: a possible function in articular cartilage. (1988) FEBS Letts **234**, 79-82

Wotton, S.F. & Duance, V.C. Type III collagen in normal human articular cartilage. (1994) Histochem. J. **26**, 412-416

Wu, C.H., Donovan, C.B. & Wu, G.Y. Evidence for pretranslational regulation of collagen synthesis by procollagen propeptides. (1986) J. Biol. Chem. **261**, 10482-10484

Wu, J-J., Lark, M.W., Chun, L.E. & Eyre, D.R. Sites of stromelysin cleavage in collagen types II, IX, X and XI of cartilage. (1991) J. Biol. Chem. **266**, (9):5625-5628

Wu, J-J., Woods, P.E. & Eyre, D.R. Identification of cross-linking sites in bovine cartilage type IX collagen reveals an antiparallel type II-type IX molecular relationship and type IX to type IX bonding. (1992) J. Biol. Chem. **267**, 23007-23014

Wu, J-J. & Eyre, D.R. Structural analysis of cross-linking domains in cartilage type XI collagen. (1995) J. Biol. Chem. **270**, 18865-18870

Yada, T., Suzuki, S., Kobayashi, K., Hoshino, T., Horie, K. & Kimata, K. Occurrence in chick embryo vitreous humor of a type IX collagen proteoglycan with an extraordinarily large chondroitin sulfate chain and short α1 polypeptide. (1990) J. Biol. Chem. **266**, 6992-6999

Yada, T., Arai, M., Suzuki, S. & Kimata, K. Occurrence of collagen and proteoglycan forms of type IX collagen in chick embryo cartilage. (1992) J. Biol. Chem. **267**, 9391-9397

Yamada, K.M. (1991) in "Cell Biology of Extracellular Matrix" (Hay, E.D. ed.), Fibronectin and other cell interactive glycoproteins. pp. 111-146, Plenum Press, New York

Yamagata, M., Suzuki, S., Akiyama, S.K., Yamada, K.M. & Kimata, K. Regulation of cell-substrate adhesion by proteoglycans immobilised on extracellular substrates. (1989) J. Biol. Chem. **264**, 8012-8018

Yang, C., Bodo, M., Notbohm, H. & Muller, P.K. Fulvic acid disturbs processing of procollagen II in articular cartilage of embryonic chicken and may also cause Kashin-Beck disease. (1991) Eur. J. Biochem. **202**, 1141-1146

Zhidkova, N.I., Brewton, R.G. & Mayne, R. Molecular cloning of PARP (proline/arginine-rich protein) from human cartilage and subsequent demonstration that PARP is a fragment of the NH_2-terminal domain of the collagen α2(XI) chain. (1993) FEBS Letts **326**, 25-28

Zhidkova, N.I., Justice, S.K. & Mayne, R. Alternative mRNA processing occurs in the variable region of the pro-α1(XI) and pro-α2(XI) collagen chains. (1995) J. Biol. Chem. **270**, 9486-9493